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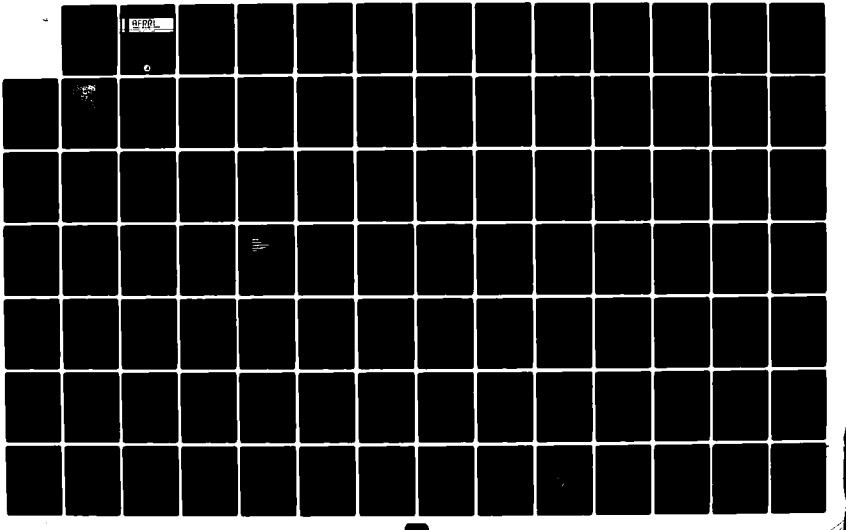

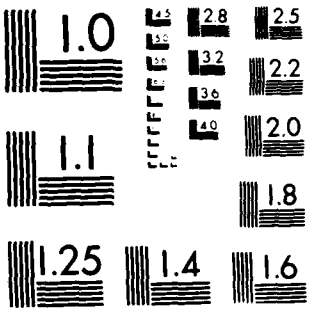

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ANNUAL RESEARCH REPORT

1 OCTOBER 1980 - 30 SEPTEMBER 1981

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ARMED FORCES RADIOBIOLOGY RESEARCH INSTITUTE

Research was conducted according to the principles enunciated in the "Guide for the Care and Use of Laboratory Animals," prepared by the Institute of Laboratory Animal Resources, National Research Council.

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This report contains a summary of the research projects of the Armed Forces Radiobiology Research Institute for the period 1 October 1980 through 30 September 1981.

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CONTENTS

INTRODUCTION

The Armed Forces Radiobiology Research Institute was established in 1961 as a subordinate command of the Defense Nuclear Agency. It is the primary Department of Defense facility for scientific research in the field of radiobiology and related matters. It conducts applied and basic research that is essential for the operational and medical support of the Department of Defense. The work is carried out by five scientific departments as listed below:

Behavioral Sciences: Effects of ionizing radiation, chemicals, and drugs on performance.

Biochemistry: Elucidation of mechanisms of injury, repair, and protection from the effects of ionizing radiation alone or in combination with other agents; development of improved methods to detect and quantify the severity of radation injury.

Experimental Hematology: Investigation of radiation injury of bone marrow; development of therapy for damage from intermediate radiation doses; determination and treatment of injuries caused by combined effects of radiation, blast, and burns.

Physiology: Research on cellular, tissue, and whole-animal models to determine physiological and biophysical changes resulting from radiation either alone or in combination with drugs or other chemicals.

Radiation Sciences: Operation, maintenance, and quality control of all AFRRI radiation sources; radiation dosimetry and estimation of tissue doses at various depths in different kinds of tissues; development and use of nuclear medicine and magnetic spectroscopic techniques for determining radiation damage in animals and model systems.

The results of this broad multidisciplinary program are summarized in this report. In addition, much of the work is published in the scientific literature, where it contributes significantly to the body of radiobiological knowledge, as well as in AFRRI scientific and technical reports.

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BEHAVIORAL SCIENCES DEPARTMENT

The Behavioral Sciences Department conducts investigations assessing the effects of ionizing radiation, chemicals, and drugs on combat performance. This program encompasses a spectrum of multidisciplinary approaches in experimental animal models, ranging from operant conditioning techniques to methods used in physiological psychology, neurochemistry, and neurophysiology. The Department addresses the specific behavioral consequences of exposure to ionizing radiation, and explores the biological mechanisms responsible for radiation-induced behavioral decrements. Collaborative efforts are pursued with other AFRRI departments, the Naval Research and Development Command, and the National Institutes of Health.

The Department has two Divisions: Experimental Psychology and Physiological Psychology. The Experimental Psychology Division uses behavioral and electrophysiological models to determine the conditions under which ionizing radiation and militarily relevant chemicals can degrade combat performance. Behavioral tasks that model specific aspects of cognitive and physical combat performance are used to determine the radiation levels that affect combat effectiveness. The information from these studies is compiled into a data base that provides rapid retrieval, overall summary analyses, and development of extrapolated models applicable to battlefield conditions. Research in behavioral toxicology quantitates the changes in behavioral and neurophysiological capabilities due to exposure to chemicals and radiations that may be present in the military environment. This work uses a battery of tests designed to provide information on toxic dose levels and/or mechanisms by which these environmental hazards affect behavior.

The Physiological Psychology Division explores the mechanisms by which ionizing radiation and chemical toxins disrupt behavior. Behavioral, physiological, and neurochemical approaches are predominant. This information can be used to develop methods of preventing performance decrement.

The results obtained from the research of this Department are disseminated to the military services and appropriate government agencies by means of informal reports, committee assignments, working groups, and correspondence. Information is also transmitted through publication in the open scientific literature and through presentation at scientific meetings.

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RELATIVE EFFECTIVENESS OF HIGH-ENERGY AND FISSION SPECTRUM NEUTRONS IN PRODUCING BEHAVIORAL INCAPACITATION, EMESIS, AND MORTALITY IN THE MONKEY

Principal Investigator: R. W. Young
Collaborator: D. M. Kunihiro
Technical Assistance: P. Mannon and J. R. Harrison

The relative effectiveness of high-energy and fission-spectrum neutrons in producing behavioral incapacitation, emesis, and mortality was compared in a series of studies with the monkey (Macaca mulatta) (1). For these studies, behavioral incapacitation was defined as cessation of response for at least 1 minute to a shock-avoidance, paired visual discrimination task. Only productive retching was scored as emesis. Comparisons of mortality were based on survival times.

In the first series of these studies, the response of monkeys on these three biological end points was compared after head-only irradiation by a cyclotron-generated high-energy neutron field obtained by using the Be9(d,n)B10 reaction from 34.7-MeV deuterons, and a bremsstrahlung field obtained by bombarding a 110-ml tantalum target with a collumated 13-MeV beam from the AFRRI linear accelerator. These studies used head-only irradiations because of the limited beam size from the cyclotron. Bremsstrahlung was used as the reference radiation field to match the depth dose distribution from the cyclotron.

In the second series of studies, the effectiveness of whole-body radiations was compared by using a fission-spectrum neutron field generated with 6 inches of lead between the subject and the AFRRI TRIGA reactor core, and a fission-spectrum gamma field obtained from the TRIGA by thermalizing the neutrons from the reactor core with 5 inches of water.

The results of these two studies clearly support the previously reported observation that neutrons are significantly less effective than gamma photons in producing behavioral incapacitation. The neutron relative biological effectiveness (RBE) for behavioral incapacitation was calculated to be 0.68 in the fission-spectrum field and 0.56 in the high-energy field. Because the high-energy neutron exposures were head-only and the fisson-spectrum exposures were total-body, these data suggest that high-energy neutrons are

no more effective in producing behavioral incapacitation than are fission-spectrum neutrons, and they may be less effective if whole-body exposures are compared. However, the neutron fields were much more effective than the photon fields in producing vomiting and death. The neutron RBE's for these end points were all greater than 1.4. Particularly noteworthy is the observation that for the head-only irradiations, some animals vomited in all of the neutron dose groups but in none of the photon groups.

The observations within the head-only and whole-body studies are consistent with one another, and they strongly suggest that neutrons are (a) less effective than photons in producing incapacitation and (b) more effective in producing emesis and death. Notwithstanding, the relative toxicity of these two radiation fields can be definitively addressed only with a high-energy neutron field large enough to deliver a total-body irradiation.

REFERENCE

1. Young, R. W., and Kunihiro, D. M. The relative effectiveness of high-energy and fission-spectrum neutrons in producing behavioral incapacitation, emesis, and mortality in the monkey. Radiation Research 83: 415, 1980.

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DATA BASE DEVELOPMENT AND DATA ANALYSIS FOR RADIATION-INDUCED PERFORMANCE DECREMENT IN PRIMATES

Principal Investigators: R. W. Young, C. G. Franz, S. G. Levin, and W. F. Jackson
Collaborators: W. E. Mitchell and D. O. Norman
Technical Assistance: J. R. Harrison, A. B. Butler, L. Clark, and P. Mannon

Over the past 15 years, the Behavioral Sciences Department has conducted a series of studies on the effects of ionizing radiations on the behavior of primates, using the monkey as a model for the human response to irradiation. In most of these studies, the behavioral parameters that were measured were held constant while the radiation parameters were varied. This approach has produced a large body of data on the acute behavioral response to ionizing radiations as a function of dose, dose rate, dose fraction, radiation field, and partial-body exposure for incapacitating range of radiation doses.

A major analytical effort was undertaken to produce a complete family of behavioral incapacitation curves as a function of both time after irradiation and total body dose. This type of comprehensive analysis was necessary to permit the use of these data in the dynamic combat effectiveness models used by the Army to predict the impact of radiation on military effectiveness. The data were taken from the AFRRI computerized Primate Data Base (1). Since this approach used data derived from several experiments conducted over a period of 10 years in two different radiation fields using two different exposure rooms, the first step in applying these primate data to man in the battlefield was the thorough reevaluation and verification of the radiation dosimetry.

Several steps are required to accurately convert reactor primate exposures to free-in-air equivalent for man. First, an accurate tissue dose and neutron-gamma ratio at the midhead of the monkey must be determined for the reactor exposures. Second, accurate free-in-air battlefield doses and radiation spectra have to be estimated. Third, battlefield spectra have to be folded into a model of man's head to obtain tissue doses and neutron-gamma ratios for man in the battlefield. With these steps completed, one can determine the midhead dose for man that equals the midhead dose for monkey. This midhead dose for man can then be used to determine the equivalent free-in-air dose for man for a given radiation effect.

In order to accurately determine tissue doses and neutron-gamma ratio at the midhead of the monkey for the neutron-gamma 4:10 and 3:1 reactor exposure fields, several steps were taken. An extensive series of measurements were made in the two fields to determine the radiation levels inside monkey phantons and free in air. The dosimetry coefficients used to determine the dose from the radiation detectors in the exposure rooms were checked, using the theoretical work done by Science Applications, Incorporated (2), in mapping the radiation field using Morse Monte Carlo Transport Codes and the 1D Discrete Ordinate Transport Codes. The results of these two effects were compared with estimates of dose to the midhead of the monkey in the AFRRI reactor, obtained from 1D radiation transport calculations performed by Oak Ridge National Laboratory (ORNL). The final step in relating tissue dose for the monkey in the reactor to free-in-air doses for man in the battlefield is to be provided by the radiation transport calculations from ORNL to estimate free-in-air dose to the midhead of man from "typical" nuclear weapons.

The analysis to relate combat ineffectiveness to these radiation doses has begun. This analysis will account for combat ineffectiveness due to early transient incapacitation, permanent complete incapacitation, and death on a minute-by-minute basis during the first 24 hours after irradiation. It is projected that this analysis, when completed, will provide distributions for the onset, frequency, and duration of incapacitating events for up to 2 weeks after exposure.

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2. Verbinski, V. V., Cassapakis, C. G., Ferlic, K. P., and Daxon, E. Calculations of the neutron and gamma ray environment in and around the AFRRI TRIGA reactor. Final Report SAI-272-[illegible]-83R1-LJ. Science Applications, Inc., McLean, Virginia, June 1981.

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ACUTE TOXICITY OF PETROLEUM-DERIVED AND SHALE-DERIVED JP5

Principal Investigators	V. Bogo, R. W. Young, and G. A. Parker, *AFRRI*
Collaborator	T. A. Hill, *Naval Medical Research Institute*
Technical Assistance	C. A. Bowand, G. G. Kessell, and C. L. Feser, *AFRRI*

Rats were gavaged with 60, 48, 38, 30, or 24 ml/kg body weight of either petroleum-derived jet propulsion fuel number 5 (JP5) or one of three samples of shale-derived JP5 (1). The surviving rats were sacrificed at 14 days after dosing. In another study, rats were gavaged with one of the four fuel samples at the rate of 24 ml/kg body weight and sacrificed at 1, 2, or 3 days postdosing.

A significant difference was seen in the lethality of the three shale-derived samples, even though all originated from the same shale deposit. The difference in toxicity was attributed to variations in the chemical composition of the crude oil in refining processes. Elevated packed cell volume and red cell count on days 1 and 2 indicated severe dehydration. White cell counts were markedly reduced on days 1 and 2. All hematologic parameters were similar to control values by 3 days after dosing. Pathologic findings in rats that had died within 24 hours of dosing indicated cardiovascular collapse. Rats that had died later showed change in hepatic periportal fat, formation of renal hyaline droplets, and fatty change. Figure 1 exemplifies the cytoplasmic vacuolization in periportal hepatocytes.

Rats sacrificed at daily intervals following the administration of JP5 (24 ml/kg) had moderate renal and hepatic functional alterations, as indicated by serum chemistry determinations. Renal hyaline droplet formation and fatty change were microscopically evident on all 3 days, but hepatic fatty change was not present until day 2.

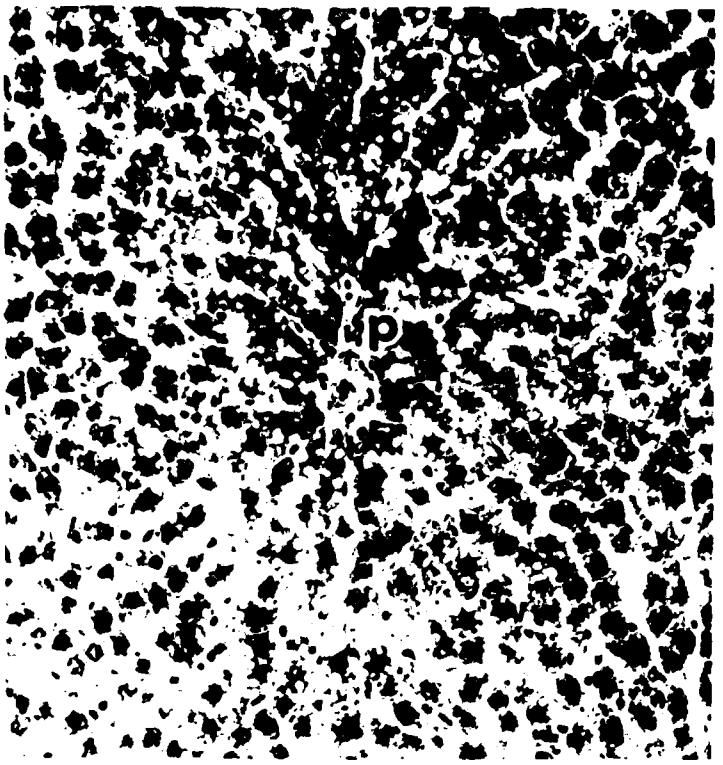

Figure 1. Cytoplasmic vacuolization in periportal hepatocytes in rat sacrificed 48 hours after gavage with 24 ml/kg refined shale JP5. Note sparing of hepatocytes away from portal triad (P). Paraffin embedded. H & E. X 210.

REFERENCE

1. Parker, G. A., Bogo, V., and Young, R. W. Acute toxicity of conventional versus shale-derived JP5 jet fuel: Light microscopic, hematologic, and serum chemistry studies. Toxicology and Applied Pharmacology 57: 302-317, 1981.

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BEHAVIORAL AND NEUROPHYSIOLOGICAL TOXICITY OF PETROLEUM AND SHALE JP5

Principal Investigators: V. Bogo, R. W. Young, and R. M. Cartledge, *AFRRI*
T. A. Hill, *Naval Medical Research Institute*
Collaborators: C. L. Feser and J. Nold, *AFRRI*
L. L. Pitts, *NMRI*
Technical Assistance: C. A. Boward, S. L. Hargett, L. Heman-Ackah, G. G. Kessell, and M. Sanders *AFRRI*

The toxicity of petroleum- and Sohio shale-derived jet propulsion fuel number 5 (JP5) was evaluated in a series of acute gavage and subchronic inhalation studies with rats. Gavage experiments at doses from 3 to 24 ml/kg were performed to test for alterations in behavior. Inhalation studies were conducted to assess behavioral and neurophysiological toxicity at concentrations from 700 to 1600 mg/m^3. Behavioral evaluation included measuring home cage activity, food and water consumption, weight, general behavior, aggression, and motor integration. Neurophysiological function after inhalation exposure to Sohio JP5 was evaluated by using somatosensory evoked potentials.

Behavioral changes noted after the oral administration of both petroleum and Sohio shale JP5 included (a) a marked transitory increase in overnight home-cage activity, and (b) a decrease in weight and in consumption of food and water for up to 3 days after dosing. The increased activity produced by petroleum JP5 lasted from $2\frac{1}{2}$ to 5 hours after dosing. Since no corresponding change in performance was seen on a motor integration task, the home-cage hyperactivity seemed more related to irritation than to any specific neurotoxicity. Some subjects exposed to petroleum JP5 were hypersensitive to touch, a few mutilated their tails, and others became aggressive. However, this aggressiveness could not be confirmed when measured with a specific-function test.

During the inhalation studies, significant increases in water consumption occurred with both fuels, beginning on the 8th day of exposure and continuing throughout the studies (Figure 1). This suggests that (a) the renal toxicity of JP5 after oral administration [which we reported earlier (1)] may also be seen after inhalation exposure, and (b) its occurrence is independent of product origin, refinery process, or route of administration. No other obvious changes were seen in the remaining behavioral tests or the evoked potentials.

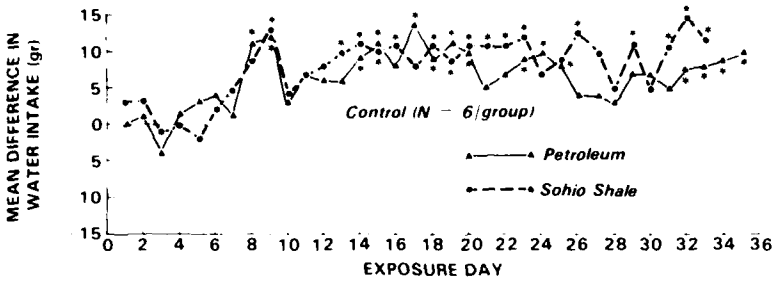

Figure 1. Water intake of rats (n = 6/group) inhalation-exposed to petroleum or Sohio shale JP5 for 6 hours/day and 5 days/week for more than 30 days. Profiles are daily calculated mean differences between control and exposure conditions. No intake differences occurred before testing. Exposure concentrations in petroleum and shale studies were 1125 mg/m^3 as decane and 1635 mg/m^3 nominal, respectively. * shows significant effects ($p < 0.05$).

REFERENCE

1. Parker, G. A., Bogo, V., and Young, R. W. Acute toxicity of conventional versus shale-derived JP5 jet fuel: Light microscopic, hematologic, and serum chemistry studies. Toxicology and Applied Pharmacology 57: 302-317, 1981.

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AN AUTOMATED METHOD FOR DETERMINATION OF BIOGENIC AMINES AND THEIR METABOLITES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

Principal Investigators: T. K. Dalton and W. A. Hunt

For several years this laboratory has been investigating the role of neurotransmitters in the underlying mechanisms of behavioral decrements induced by ionizing radiation. Initial studies indicate that during early transient incapacitation the potassium-stimulated release of dopamine from slices of the caudate nucleus is significantly enhanced (1). Our desire to examine this effect in greater detail, including its possible significance to other areas of the brain, was impaired because of the lack of a suitably sensitive method that would allow the measurement of various biogenic amines and their metabolites in a single tissue sample. Such measurements would provide an index of the degree of activity of neurons, using dopamine, norepinephrine, and serotonin as transmitters. Therefore, we began to develop a method that would allow the analysis of a variety of biogenic amines and their metabolites with maximum productivity.

A procedure has been established using high-performance liquid chromatography that is fully automated and will allow the analysis of up to 200 samples per week. The following compounds can be separated and quantitated in the order that they appear on the chromatogram: dihydroxymandelic acid (DOMA), vanilmandelic acid (VMA), dihydroxyphenylglycol (DHPG), dihydroxyphenylalanine (L-DOPA), norepinephrine (NE), 3-methoxy-4-hydroxyphenylglycol (MHPG), epinephrine (EPI), dihydroxyphenylacetic acid (DOPAC), 5-hydroxytryptophan (5-HTP), dopamine (DA), 5-hydroxytryptophol (5-HTOH), 5-hydroxyindoleacetic acid (5-HIAA), homovanillic acid (HVA), 3-methoxytyramine (3-MT), and serotonin (5-HT). Only VMA and DHPG are not well resolved and elute off the column together. Figure 1 is a sample chromatogram.

The method promises to allow the acquisition of considerable data in a short time, and it will be useful for analyzing various areas of the brain and biological fluids.

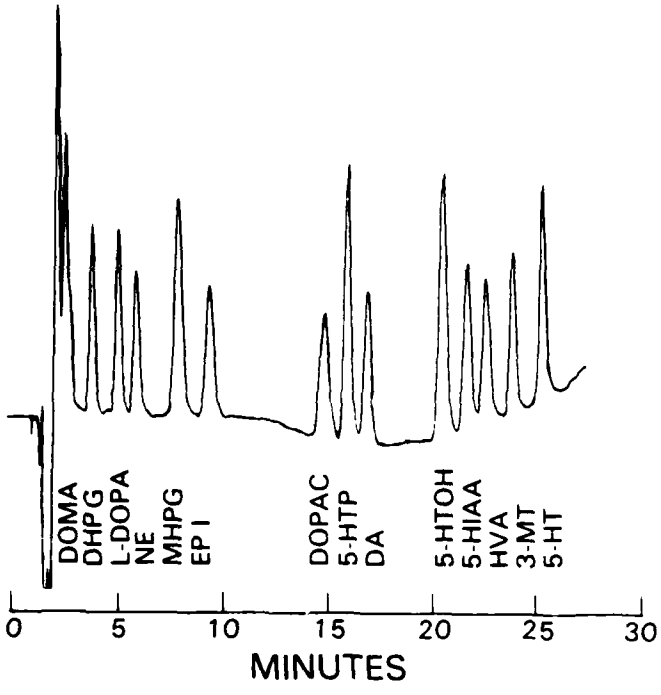

Figure 1. Chromatogram of 5-pmole standards eluted from a C18 reverse-phase column using a 100-mM potassium phosphate buffer (pH 3.5) containing 4 mM 1-heptanesulfonic acid, 100 μM EDTA, and increasing concentrations of acetonitrile (1%-20%). Analysis time was 30 minutes.

REFERENCE

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ROLE OF HISTAMINE IN RADIATION-INDUCED CONDITIONED TASTE AVERSION

Principal Investigators: B. M. Rabin, *Department of Psychology, University of Maryland, Baltimore, Maryland*
W. A. Hunt, *AFRRI*
Technical Assistance: J. F. Lee

Nausea and vomiting are major signs of exposure to moderate doses of ionizing radiation. Studying the mechanisms of the development of these signs by using dogs or monkeys can be cumbersome and expensive. The use of small laboratory animals, such as rodents, would be much more practical, but they do not vomit. To circumvent this problem, a model system could be used to provide valuable information on the mechanisms of emesis, at lower cost and higher productivity.

One such model is the conditioned taste aversion (CTA). When a novel-tasting solution, normally liked by the animal, is presented with a toxic stimulus, the animal associates the consequence of this stimulus with the consumption of the tasting solution, and subsequently avoids it. Using this paradigm, the possibility that histamine might be a causative factor in the development of a radiation-induced CTA was examined. Histamine had been implicated by a study in which CTA learning was reported to be blocked by antihistamine pretreatment (1).

The first set of experiments studied the ability of histamine to induce a CTA when injected intraventricularly. This design was used to minimize the involvement of peripheral mechanisms. Histamine was capable of inducing a CTA that could be antagonized by H1 blockers but not H2.

Although histamine could possibly be a mediator in the development of radiation-induced CTA, the next set of experiments indicated that antihistamines were ineffective antagonists of it. Also, tolerance to repeated doses of histamine had no effect. These data do not support the hypothesis that histamine is involved in radiation-induced CTA learning.

REFERENCE

1. Levy, C. J., Carroll, M. E., Smith, J. C., and Hofer, K. G. Antihistamines block radiation-induced taste aversions. Science 150: 1044-1046, 1974.

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INTERACTIVE EFFECTS OF STRESS AND RADIATION ON THE PREFERENCE AND USE OF INFORMATION

Principal Investigator: W. F. Burghardt, Jr.
Technical Assistance: B. A. Dennison

Previous research at AFRRI and elsewhere has indicated that supralethal doses of ionizing radiation can profoundly disrupt performance on a number of behavioral tasks. Heretofore unexplored was the specific question of the nature of the motor and cognitive changes that occur in these situations. Although the underlying biological mechanisms of performance decrement are being explored in other laboratories, the importance of environmental cues in maintaining performance after irradiation has not been investigated.

In order to better interpret the alterations in behavior due to changes in the use of stimuli after irradiation, a task has been developed using the rat, which may provide some evidence of an organism's preference for and use of informational stimuli in an aversively motivated task. Preliminary information indicates that this task is stable over time and is responsive to a number of environmental manipulations. Irradiation appears to induce a performance decrement on the avoidance component consistent with decrements obtained in other situations. The animal's preference for informational cues also appears altered, although the animal seems able to use those cues when they are present.

Further research will be undertaken to verify these findings, and to determine a dose-response relationship for this effect. Application will be made to a primate model after rodent validation. This paradigm should also be useful in determining the combined effects of stress and radiation.

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BIOCHEMISTRY DEPARTMENT

The main objectives of the research conducted in the Biochemistry Department are (a) elucidation of the biochemical mechanisms of injury, repair, and protection of the mammalian organism from the effects of radiation, either alone or in combination with other toxic agents, and (b) development of more effective, sensitive, and easy-to-perform methodologies to detect and quantify the severity of radiation-induced injury in man.

The Department is organized into three Divisions: Physiological Chemistry, Molecular Biology, and Immunological Chemistry.

The Physiological Chemistry Division is primarily concerned with the identification and development of biochemical indicators of radiation injury. Major emphasis is being placed on changes in serum glycoproteins, trace metals, and indicators of radiation-induced membrane lipid peroxidation. The effects of radiation on the immune response of the mammalian organism have been investigated. In addition, extensive examination has been made of the role of various immunostimulants alone or in combination with radioprotectant drugs in alleviating or preventing radiation-induced injury. Various dietary supplements such as vitamins E, C, and A are being tested for their radioprotective properties. The effect of ionizing radiation on bone and bone marrow formation has been studied.

The research objective of the Molecular Biology Division is the clarification of the biochemical mechanisms of injury that has been induced by toxic agents such as ionizing radiation alone or in combination with toxic chemicals, especially commercially available anticholinesterases. Methods for the more effective protection of the mammalian organism from the effects of these agents are under extensive investigation. Identification of the sequence of biochemical events leading to radiation-induced damage to cellular membranes and the role of various radioprotectants in preventing or alleviating this damage are also being studied. The effects of radiation on histamine and prostaglandin release and the mechanisms responsible for this release are being systematically examined.

The Immunological Chemistry Division is concerned with the isolation of hematopoietic and progenitor cells and the measurement of their potential as a modifier of radiation-induced injury. The nature of the interaction of stromal tissue with hematopoietic cells and its importance in postirradiation hematopoietic regeneration has also been investigated.

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EFFECT OF CALMODULIN ON HISTAMINE RELEASE IN THE RAT MAST CELL

Principal Investigators: D. L. McClain, M. A. Donlon, and G. N. Catravas
Technical Assistance: W. W. Wolfe and K. T. Lambright

Ionizing radiation causes a massive release of histamine from peripheral mast cells. This response to a 4000-rad dose of mixed gamma-neutron radiation has been linked to postirradiation hypotensive effects and early transient incapacitation (1).

Considerable experimental evidence suggests that increases in the concentration of intracellular free calcium is critical for histamine release in the rat mast cell (2). Stimuli for mast cell degranulation act at the cell membrane to initiate a series of biochemical events that lead to an increase in cytosolic calcium. All mechanisms for calcium control involve cellular membranes and membrane-associated proteins. Radiation can lead to oxidation of protein sulfhydryl groups and peroxidation of lipids. Both can alter the permeability characteristics of the membrane and the membrane's ability to use the fine control of calcium necessary for histamine release.

Recent evidence suggests that the multiple functions of calcium in secretion may involve calmodulin, an intracellular calcium-binding protein thought to mediate many of the effects of calcium in cellular processes. In preliminary work to examine the effect of calmodulin on the mast cell, rat peritoneal mast cells were first purified by sedimentation through an albumin gradient. Addition of purified calmodulin (10^{-5} to 10^{-9} M) induced no increase in histamine release. The ability of calmodulin to modulate histamine release was tested by using the histamine-releasing agents 48/80 and concanavalin A. Calmodulin added simultaneously produced a dose-related inhibition of 48/80 with a 50% inhibition dose (ID 50) of 3-7 x 10^{-7} M (Figure 1) (3). Preincubation of the mast cells with calmodulin failed to alter the ID 50 for 48/80. However, concanavalin A (50 or 100 µg/ml) was not affected by the same concentrations of calmodulin.

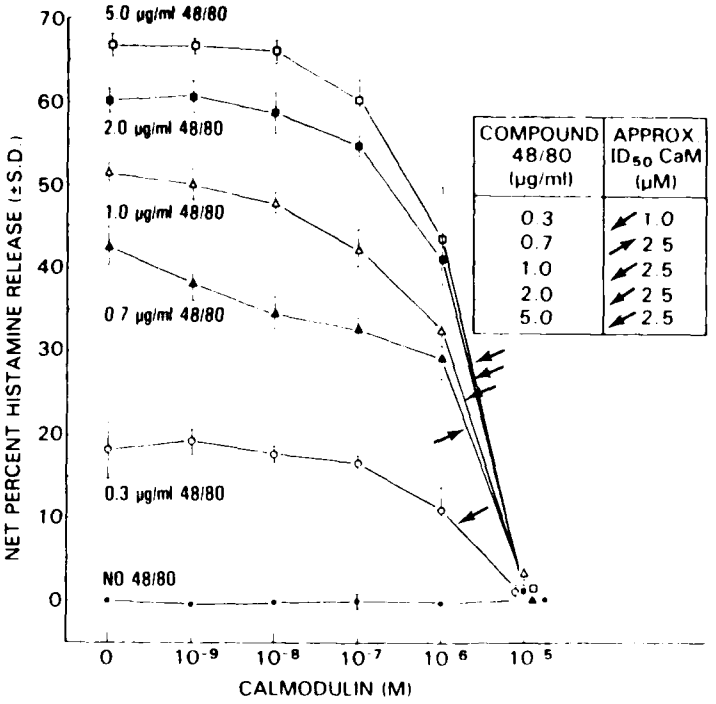

Figure 1. Effect of calmodulin (CaM) on compound 48/80-directed histamine release in rat mast cells. Purified cells were simultaneously exposed to 48/80 and CaM at the indicated concentrations. The table shows approximate concentration of CaM required to produce 50% inhibition (ID_{50}) at each concentration of 48/80.

The different mechanisms by which calmodulin affects histamine release remain to be elucidated. A possible explanation is that the calmodulin is interacting specifically with the 48/80 receptor on the cell membrane and is not acting intracellularly. Further studies will focus on calcium metabolism in these inhibited cells to investigate any relationship between calmodulin and the altered calcium metabolism seen in irradiated mast cells.

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RADIATION-INDUCED CHANGES IN PRODUCTION OF PROSTAGLANDINS $F_{2\alpha}$, E, AND THROMBOXANE B_2 IN PARENCHYMAL LUNG TISSUES OF GUINEA PIG

Principal Investigators: L. K. Steel and G. N. Catravas

The pharmacological properties of the prostaglandins, coupled with their enhanced formation and release in all types of inflammatory reactions, suggest a role for these chemical mediators in the development of radiation-induced tissue injury. Because the lung is a major site of prostaglandin production, uptake, and inactivation, the present investigation was designed to examine the effects of sublethal doses of ^{60}Co gamma radiation on the generation of parenchymal lung tissue $PGF_{2\alpha}$, PGE, and TxB_2. Also investigated were the capacity of lung tissues to generate prostaglandin in response to (a) H-1 receptor stimulation by the exogenous addition of histamine, or (b) carboxylic acid ionophore A23187 stimulation of divalent cation transport.

At 1 hour to 4 days after total-body exposure of guinea pigs to 50, 150, or 300 rads gamma radiation, changes were detected in the concentrations of basal prostaglandin and thromboxane in parenchymal lung tissues (Figure 1). At 1-3 hours after exposure, tissue levels of $PGF_{2\alpha}$, PGE, and thromboxane B_2 were significantly elevated in animals receiving 300 rads, with the degree of elevation revealing a radiation dose effect. By 24 hours, tissue prostaglandin and thromboxane levels returned to near control values. Lung tissue synthesis of prostaglandins in response to H-1 receptor stimulation by the exogenous addition of histamine revealed similar radiation dose effects. Stimulation of divalent cation transport with ionophore exogenously applied to lung tissues revealed a transient peak of increased sensitivity to stimulation for TxB_2 synthesis at 24 hours and for $PGF_{2\alpha}$ at 72 hours postirradiation.

The data suggest that significant alterations in prostaglandin and thromboxane concentrations in parenchymal lung tissues occur following irradiation, and that these changes are dose-related. Altered responsiveness to H-1 receptor stimulation and divalent cation transport also occur.

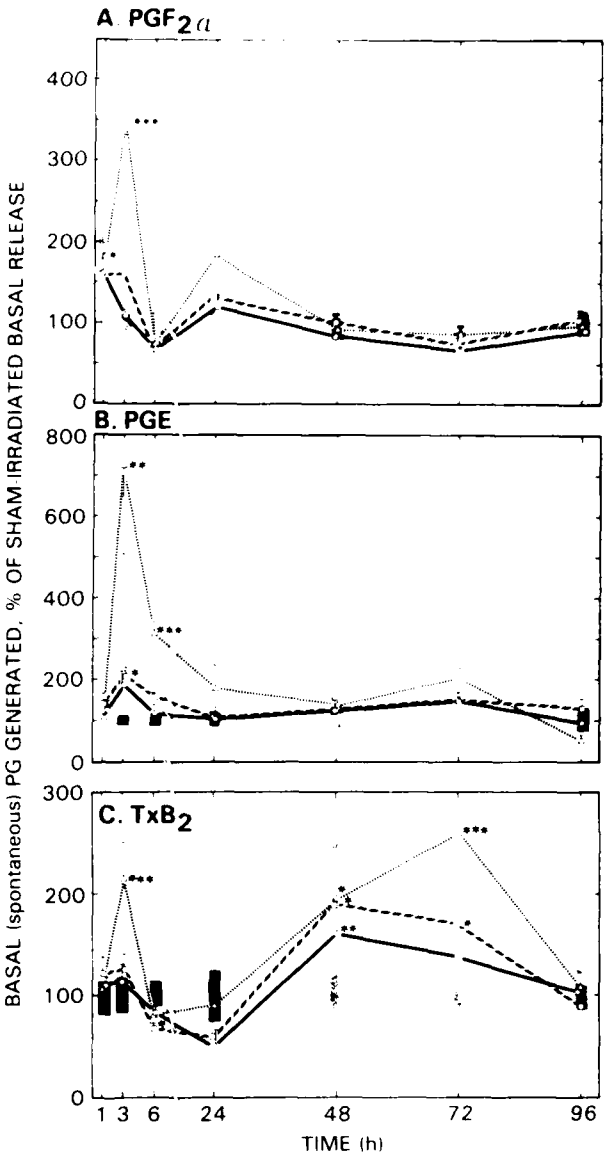

Figure 1. Effect of total-body ^{60}Co gamma radiation on basal prostaglandin levels in guinea pig parenchymal lung tissue. Parenchymal fragments were incubated in Tyrode's buffer only. Prostaglandin generation is expressed as percent of sham-irradiated (control) release at indicated times postirradiation (± 15 min). (A) $PGF_{2\alpha}$-generated; (B) PGE-generated; (C) TxB_2-generated. Control (sham) release values (pg mg lung protein) ranged from 150 to 221 pg $PGF_{2\alpha}$, 55-78 pg PGE, and 1587-1932 pg TxB_2. Individual control values at each time for each individual prostaglandin are presented as shaded (▒) bars; they did not statistically differ from one another throughout investigation. (— —), 0.5 Gy; (- - -), 1.5 Gy; (— —), 3.0 Gy. Means + SEM are given. SE indicated by vertical lines. Significantly different from controls: *$p < 0.05$; **$p < 0.025$, ***$p < 0.005$. n = 12.

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EFFECT OF RADIOPROTECTANT WR 2721 ON CYCLIC NUCLEOTIDES, PROSTAGLANDINS, AND LYSOSOMES

Principal Investigators — P. J. Trocha and G. N. Catravas

WR 2721 has been found to be one of the best radioprotective drugs against gamma radiation, and it is thought to confer its radioprotective characteristics by acting as a free radical scavenger. However, only a limited number of biochemical studies have been made on this compound or similar amino-sulfhydryl agents. The biochemical investigations that have been carried out show that these radioprotective compounds interact with various mammalian enzymes, alter several of the constituents of the immune system, affect levels and repair capacity of DNA, and can alter prostaglandin synthesis in vitro.

Within 1 hour after intraperitoneal injection of the radioprotectant WR 2721 into rats, splenic cGMP levels dropped and remained suppressed for 6 hours before returning to normal. However, if rats were exposed to ionizing radiation at 30-40 minutes after WR 2721 treatment, they had higher cGMP levels at 3 hours postirradiation than did nonirradiated, drug-treated controls, but lower cGMP content than the irradiated nondrug-treated controls. Radiation exposure of animals pretreated with WR 2721 also resulted in higher liver and spleen levels of cAMP and additional elevations in spleen prostaglandin content, compared with irradiated controls at 3-6 hours after radiation treatment.

The secondary fluctuations of lysosomal enzyme activities, prostaglandin content, and cyclic nucleotide levels were also altered in irradiated rats pretreated with WR 2721 when compared with irradiated controls. Liver and spleen lysosomal β-glucuronidase activities, spleen cAMP and cGMP levels, and spleen prostaglandin concentrations were closer to physiological levels at 3 days postirradiation in rats given WR 2721 before the radiation treatment.

The results of this study suggest that radioprotective drugs or other agents that alter levels of the components in the prostaglandin and/or cyclic nucleotide systems before or shortly after exposure to radiation may be significant factors in protecting a mammalian system against the detrimental effect of ionizing radiation.

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EFFECT OF THE RADIOPROTECTANT WR 2721 ON PROSTAGLANDIN AND LYSOSOMAL SYSTEMS IN IRRADIATED RATS

Principal Investigators: P. J. Trocha and G. N. Catravas

Prostaglandin levels, lysosomal membrane integrity, and lysosomal enzyme activities were studied in radioresistant tissues (liver) and radiosensitive tissues (spleen) taken from Sprague-Dawley rats. Enzymatic and immunoassay techniques were used to measure lysosomal β-glucuronidase activities and prostaglandin F levels in irradiated and nonirradiated rats previously injected intraperitoneally with saline or the radioprotectant S-2(3-aminopropylamino)ethyliphosphorothioic acid (WR 2721) dissolved in saline.

Rats treated with WR 2721 at 30-40 minutes before exposure to 1000 rads of ^{60}Co radiation displayed only elevated spleen prostaglandin F levels at 3-6 hours postirradiation, compared with irradiated controls given saline. At 1-3 days postirradiation, spleen lysosomal β-glucuronidase activities were lower in irradiated animals pretreated with the radioprotectant than in their corresponding irradiated controls. Also, at 3 days after radiation exposure, spleen lysosomal membrane stability and spleen prostaglandin F levels in drug-treated rats were found to be lower than in irradiated rats that had received saline. Further studies of spleen and liver tissue analyzed in animals not exposed to ionizing radiation showed that WR 2721 had no effect on the lysosomal or prostaglandin systems.

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EFFECT OF THE RADIOPROTECTANT WR 2721 ON CYCLIC NUCLEOTIDE LEVELS, LYSOSOMAL ENZYME ACTIVITIES, AND LYSOSOMAL MEMBRANE STABILITY

Principal Investigators: P. J. Trocha and G. N. Catravas

Biochemical effects of the radioprotectant S-2-(3-aminopropylamino)ethylphosphorothioic acid (WR 2721) were investigated in order to determine if this drug alters cyclic nucleotide levels and/or lysosomal systems. Using immunoassay and enzymatic techniques, liver and spleen cyclic nucleotide levels and lysosomal β-glucuronidase activities were measured in irradiated and nonirradiated rats injected intraperitoneally with saline or WR 2721 dissolved in saline.

No alterations in lysosomal β-glucuronidase activities, lysosomal membrane stabilities, or cyclic nucleotide levels were found in the spleen or liver of nonirradiated rats at 1-6 hours after injection of the radioprotectant, except for spleen cGMP content. Within 1 hour after administration of WR 2721, the spleen cGMP levels were 50% lower than their normal concentration. However, at 6 hours after injection of the radioprotectant, the spleen cGMP levels were again normal.

Animals treated with WR 2721 at 30-40 minutes before irradiation displayed a reduction in spleen lysosomal enzyme activities at 1-3 days postirradiation compared with irradiated controls given only saline. Cyclic nucleotide levels were also affected by the radioprotectant. Liver and spleen cAMP levels were found to be higher at 3-6 hours postirradiation, whereas spleen cGMP levels were lower at 1-4 hours postirradiation than the corresponding irradiated controls. At 3 days after radiation exposure, spleen lysosomal membrane stability and cyclic nucleotide levels in drug-treated rats were found to be closer to the levels of nonirradiated control rats than to those of irradiated controls receiving saline.

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CALCIUM ACCUMULATION AND RETENTION BY SYNAPTOSOMES IRRADIATED WITH HIGH-ENERGY ELECTRONS

Principal Investigators: M. J. Ely and G. N. Cafiaxis
Technical Assistance: J. M. Speicher

The effect of 14.5-MeV electrons on neuronal calcium accumulation was studied in nerve-ending particles (synaptosomes) prepared from homogenates of whole rat brain. Irradiation of synaptosomes with doses of 50, 100, or 500 Gy did not result in significant changes in calcium accumulation when measured at 3, 7, and 17 minutes after termination of exposure. Potassium-stimulated calcium accumulation was likewise unaltered by doses of up to 500 Gy.

The effect of radiation on the retention of previously accumulated calcium was also investigated. No significant changes in calcium retention were observed with doses of up to 500 Gy. Increasing or decreasing the external calcium concentration failed to elicit significant changes in calcium retention.

These findings suggest that radiation-induced changes in neuronal calcium disposition do not play a major role in the mechanisms underlying performance decrements that occur transiently and within minutes of exposure to supralethal doses of ionizing radiation.

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DNA REPAIR AND RADIOPROTECTORS: I. DNA REPAIR AS A PROBE TO RADIOSENSITIVITY AND RADIOPROTECTION

Principal Investigator: E. Riklis

The ultimate fate of an irradiated biological entity depends on various types of factors: physical (quality and dose of radiation), chemical (the ambient and physiological environment), and biological. No other factor has a greater role in determining life or death, health or disease, than the role played by the various biochemical mechanisms of repair. On a cellular level, survival of a cell population can vary by several orders of magnitude between different strains of the same family, if a strain lacks the ability to perform accurate repair of the damaged site. Long ago, immediately following the discovery of DNA excision repair (1), I stated that "the degradation of DNA which accompanied the excision of dimers in UV resistant cells, throws attention to observed degradation of DNA following x irradiation. It is possible that a general repair system exists, acting along the same lines for various kinds of radiations and in various types of cells."

Although this is still basically correct, today we recognize the complexity of the various repair mechanisms, and we know that some mammalian cells may be deficient in their repair capability because of genetic impairment. This results in increased sensitivity to radiation and to alkylating agents.

Two basic questions, related to the problem of sensitivity and risk assessment, need to be answered: (a) Since there are different individual sensitivities to radiation, can we measure and predetermine who is "at risk" from radiation? And if so, are there any preventive measures that might reduce that risk? (b) Can we change the individual radiosensitivity by protecting the genetic material or by increasing the cellular repair capabilities? And can this be achieved using chemical radioprotectors?

The search for agents to modify the effects of radiation on biological systems was begun parallel to the search for understanding the mechanisms of radiation sensitivity, with two opposite goals: selectively sensitizing cancer cells and protecting normal cells. It has long been recognized that sensitizing a cell may be achieved

by inhibiting the cellular repair mechanisms. But the studies on protection have been based on the notion that protection may be achieved by scavenging the free radicals formed by radiation, which are believed to be responsible for most of the damage. For this reason, much of the effort in the development of radioprotectors has gone into developing chemical compounds to act as radical scavengers. The end point for most of the studies (carried out on animals or on cells in culture) was lethality. Surprisingly little was done toward studying the possibility of protecting the genetic material of cells. Clearly, to be applicable to humans, a method was required that would enable in vitro determination of the radiation sensitivity of a living human. It has been suggested (2,3) that the measurement of the potential for repair of DNA damage, or the "repair capacity," will indicate the vulnerability to radiation--not only in terms of survival but also in terms of risk of carcinogenicity.

Accordingly, a relatively simple previously developed method has been modified to allow the determination of (a) the repair capacity of cells in culture and (b) the effects of radioprotectors on the repair capacity. The method is based on the complete inhibition of the semiconservative DNA synthesis, as detailed below.

Cells are grown to logarithmic phase, attached to a plastic surface in flasks or dishes. 0.01 uCi/ml ^{14}C-thymidine is added 16 hours before start of experiment. Growth medium is replaced by phosphate-buffered saline (PBS) containing 1 x 10^{-5} M trimethylpsoralen (TMP). Cells are incubated for 15 minutes in CO_2 incubator at 37°C. Cells are irradiated with near-UV light. Flux: 20 $Jm^{-2}sec^{-1}$; fluence: 10,000 Jm^{-2} with cover and 6,000 Jm^{-2} without cover. Buffer is immediately replaced by medium, and cells are incubated for 2 hours. Medium is replaced by PBS. If a radioprotector is being studied, it is added now to the cell culture and incubated for 30 minutes.

Cells are irradiated either in flasks or in petri dishes. Buffer is immediately replaced by medium containing 5 uCi/ml ^{3}H-thymidine, and cells are incubated for 90 minutes. In parallel experiments, ^{3}H-thymidine is added before irradiation. Medium is aspirated; attached cells are washed and lifted by 0.25% trypsin. Cells are collected on filters in a specially designed cell harvester, washed with ethanol, dried in air, and then placed in counting vials with toluene-based scintillation fluid.

^{3}H and ^{14}C are counted in a liquid scintillation counter, and the amount of thymidine uptake is calculated per 10^5 cpm ^{14}C, which is equal to about 10^5 cells. The number of counts of incorporated ^{3}H-TdR at a given dose divided by the number of counts of the non-irradiated cells treated with PUVA is designated as the repair capacity of the cells. Different radiation sources have been used, including the cobalt-60 source at AFRRI for gamma irradiation, the TRIGA reactor at AFRRI for neutrons, and the 18-MeV LINAC for accelerated electrons. Results describing the repair capacity of cells following gamma and accelerated-electron irradiation as well as the effect of the radioprotector WR-2721 are described in the item following.

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DNA REPAIR AND RADIOPROTECTORS: II. DESIGN OF A UNIVERSAL CELL-HARVESTING SYSTEM

Principal Investigator: E. Riklis

The method for studying the DNA repair capacity of cells in culture requires at least five changes of buffer and medium by aspiration and addition. The last step requires harvesting the treated cells onto filters for counting of radioactivity. A regular experiment required at least 10 triplicate cell cultures in 30 flasks or petri dishes. In an experiment with that number of samples, the time that elapsed from handling the first sample to the last was far too long. This could cause incorrect results because of different lengths of time that the cells were with trimethylpsoralen, without medium, with ^{3}H-thymidine, etc. Also, the several incubation periods that are required throughout an experiment meant loading and unloading flasks on trays and carrying them to the incubator either upstairs or downstairs.

Therefore, an automatic system was designed to allow (a) aspirating or adding buffer to 12 flasks or dishes at a time, (b) handling flasks, test tubes, petri dishes, and six well clusters, and (c) treating 12 flasks at a time and carrying them back for incubation without the need for loading or unloading.

The design of the harvester was based on the Harvester MS-12 of Brandel, Inc. Brandel also fabricated the new harvester that allows a simple change of probe for use with flasks or dishes by using a common point of distribution where effluents aspirated by the 12 prongs, or buffers added through 12 prongs, are passing through in closed lines that are easily separated for a change of probe.

The special flask holder for 12 flasks allows them to be placed in the incubator in a vertical position and to be taken for treatment in a horizontal position. Irradiations in the LINAC or the cobalt facility were performed with up to 21 flasks arranged in a perpex stand.

With the harvester and the holders, up to 120 cell culture flasks per experiment can be handled easily, providing the possibility of studying different doses of radiation and several other variables, within one experiment.

When six well cluster dishes were used (for UV irradiation and later for gamma and neutron irradiation), it was possible for one person to easily handle 20 clusters, thus using 120 cell cultures. With the aid of another investigator, 30 clusters (180 samples) could be used. Thus, a DNA repair experiment with three different cell lines and several variables could be performed within 1 long day.

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DNA REPAIR AND RADIOPROTECTORS: III. INCREASED DNA REPAIR CAPACITY OF CELLS BY THE RADIOPROTECTIVE COMPOUND WR-2721 FOLLOWING GAMMA AND ACCELERATED ELECTRON RADIATIONS

Principal Investigators: E. Riklis, M. P. Hagan, and G. N. Catravas
Technical Assistance: D. P. Dodgen

The main objective of this work was to devise a system to determine if the radioprotecting drugs (WR-2721 in particular) are protecting the genetic material and, if so, whether they are protecting it by reducing the damage or by affecting the cellular DNA repair mechanisms. If the radioprotecting drugs increase DNA repair, they may improve the chances of a cell to survive sublethal doses and to recover its genetic matter, thereby reducing mutagenicity and carcinogenicity.

The method of measuring DNA repair capacity is described in the preceding item. The survival of the V-79 cells in these studies was determined by counting colony formation. The resulting survival curves were similar in shape and magnitude to those reported in numerous other published works. Survival from accelerated electrons is shown in Figure 1. The relative biological effectiveness is somewhat lower than that for gamma radiation, about 0.9. The effect of WR-2721 on survival, although small, is meaningful; when corrected for concentration and period of drug exposure, it confirms the results obtained with X rays for other types of cells (1).

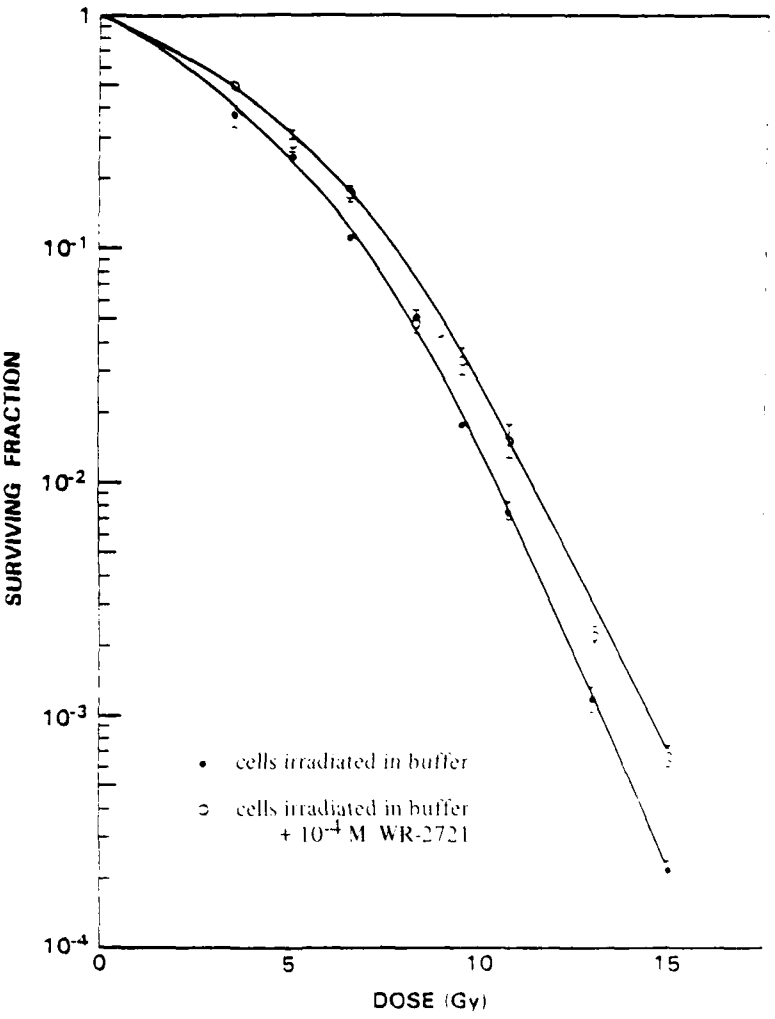

Figure 1. Survival of Chinese hamster V-79 cells from 18-MeV accelerated electron radiation

Less damage of genetic material occurs in protected cells that show increased repair capacity, thus assuring less genetic and somatic effects later.

Our attention is focused on DNA repair capability. It is measured as the ratio of the amount of ^{3}H-thymidine incorporated into DNA following a given dose, over the amount incorporated following the lowest measurable dose. The ratio at the highest dose over that at the lowest dose is the repair "capacity" of that cell. The

method of measurement is independent of residual synthetic activity or other causes of background fluctuations, since these are included in the controls only treated with trimethyl psoralen plus near-ultraviolet light.

Repair capacity is shown in Figures 2 and 3 for cells exposed to gamma and electron radiation. All cells show a certain amount of repair capability of the damage inflicted by the different radiations. The radioprotecting compound WR-2721, when present before and during irradiation, brings about an increased repair capacity, especially at higher doses. The very high repair shown following UV radiation in the presence of WR-2721 (see the following item) may reflect synergism between long patch repair and a triggered removal of the psoralen cross-links blocking semiconservative DNA synthesis. One may assume that if less damage due to radical scavenging is produced, then increased repair would not ensue. WR-2721 demonstrates the highest effectiveness at the very high doses, as measured by increased thymidine uptake.

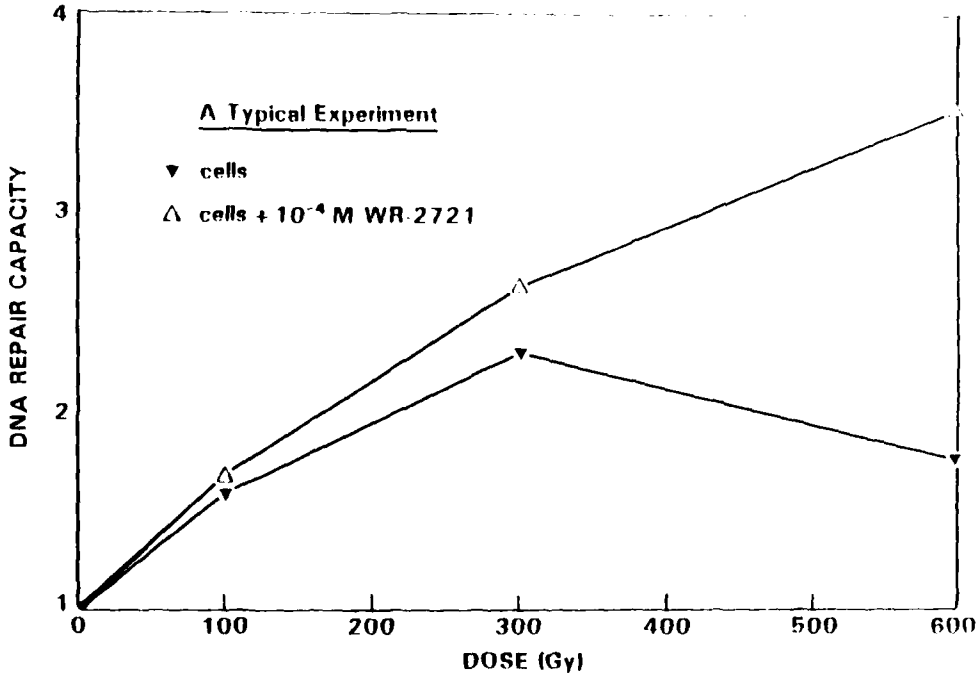

Figure 2. DNA repair capacity of gamma-irradiated Chinese hamster V-79 cells, with and without the presence of WR-2721

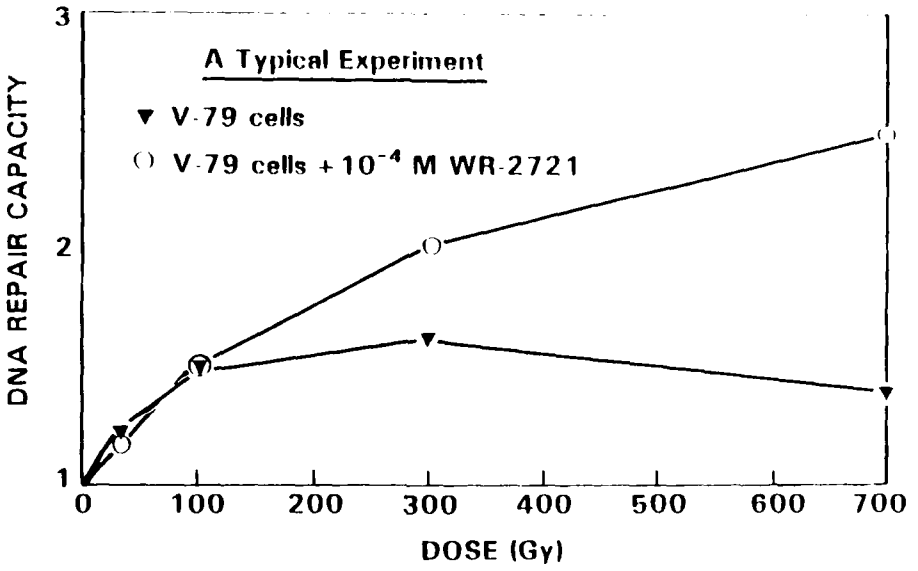

Figure 3. DNA repair capacity of Chinese hamster cells in culture following accelerated electron radiation, with and without the presence of WR-2721

It is reasonable to assume that at these very high doses, DNA damage cannot be prevented by the presence of the radioprotector. But perhaps the repair enzymes (which are otherwise fully saturated or even damaged at these doses) are better protected and thus better able to repair the DNA damage by some mechanism. This would give an overall picture of increased repair capability (2).

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DNA REPAIR AND RADIOPROTECTORS: IV. REPAIR OF MOUSE MELANOMA CELLS FOLLOWING ULTRAVIOLET IRRADIATION

Principal Investigators: F. Riklis, J. F. Preschl, and G. N. Catravas
Technical Assistance: W. N. Fry

B16/C3 mouse melanoma cells (obtained from Dr. D. L. Engelhardt, Columbia University, New York) were grown in either F-12 medium + 5% fetal calf serum (FCS), or DMEM + 10% FCS medium. The cells grown in Dulbecco's Modified Eagle's Medium (DMEM) produced melanin within 24 hours after entering the stationary phase. Cells grown in F-12 did not produce melanin for at least 4 days after entering the stationary phase. This difference in the effects of the two media on melanin production is due to differences in the bicarbonate concentrations (and therefore pH) and ionic strengths.

Ultraviolet (UV) light from a 15-W germicidal lamp was used at a flux of 2.5 $Jm^{-2}sec^{-1}$ or less. For the PUVA (trimethyl psoralen plus near-ultraviolet light) treatment, near-ultraviolet light (365 nm) was provided by tubular black-light lamps at a flux of 20 $Jm^{-2}sec^{-1}$.

The survival curves of DMEM-grown and F-12-grown cells showed about the same sensitivity to UV radiation (254 nm) in terms of D_o. But the DMEM-grown cells had a definite shoulder, signifying the repair of sublethal damage, whereas the F-12-grown cells did not demonstrate it. In measuring DNA repair capacity by the method described in the preceding papers, the DMEM cells demonstrated a higher degree of incorporation of ^{3}H-TdR, both without and with (following) UV irradiation (Figure 1).

However, the ratio of the high dose to the low dose, the repair capacity, is only slightly higher in the DMEM-grown cells. WR-2721 increased the repair capacity of the UV-irradiated cells. This is a surprising result in view of the fact that most UV damage is formed by direct damage to DNA, rather than by free radical formation. An explanation for the WR-2721-mediated increased repair may be the possible protection of the repair enzymes, and also the fact that on UV irradiation, WR-2721 forms adducts with DNA, and the repair of such adducts, recognized as damage, may contribute to the increased repair synthesis.

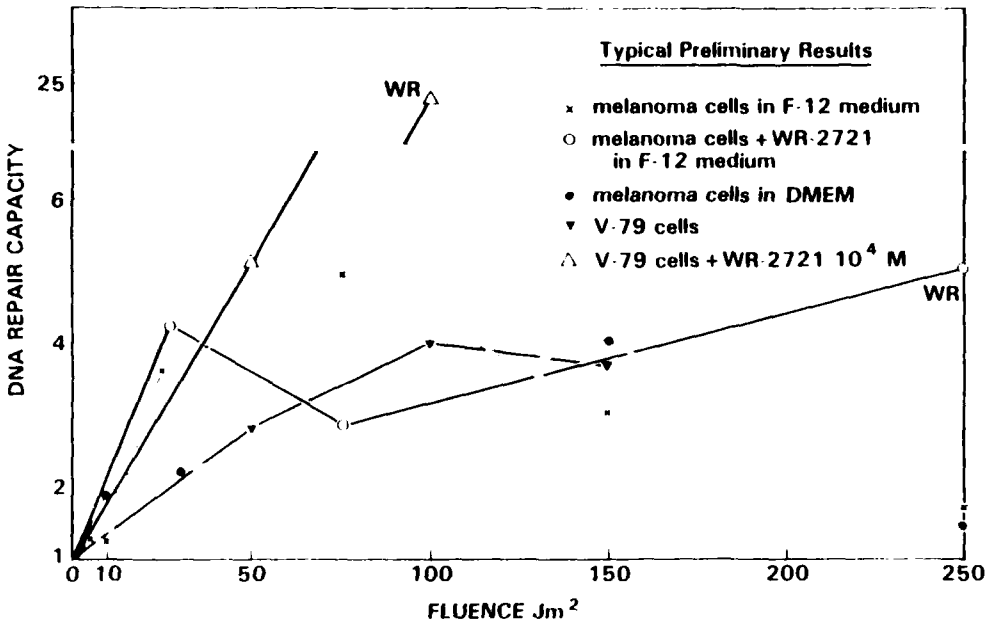

Figure 1. DNA repair capacity of cells irradiated with near-ultraviolet light

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VITAMINS AND RADIATION INJURY

Principal Investigators: V. Srinivasan, J. F. Weiss, and S. A. Simpson
Technical Assistance: L. V. Cummings, C. M. Morris, and W. W. Wolfe

The effects of portal and whole-body radiation on nutritional status and, conversely, the effects of specific dietary elements on radiosensitivity, are poorly understood. Since certain nutrients may influence immune status (e.g., vitamins C, E, and Z; zinc; pyridoxine), the role of nutrition in radioprotection will be investigated with respect to the interrelationship of radiation-induced immunosuppression and nutritional status. The effects of vitamins on the detoxifying enzyme systems important in radiation and chemical damage are also being studied.

The role of vitamin E as a radioprotector has been investigated, but the reports concerning its efficacy are conflicting. The antioxidant effect of vitamin E has been cited to explain its protective effect, but its overall role in recovery from radiation injury needs additional investigation. Further, the role of B-complex vitamins as radioprotectors needs to be studied. In the present study (1), male mice (C57BL/6J or CD2F1), 6-7 weeks old, were fed diets containing higher amounts (three times the NAS/NRC recommended daily allowance) of either vitamin E or vitamin B_6 for 1 week before cobalt-60 irradiation. Survival was investigated for a 30-day period on the same diets.

Vitamin B_6 had no protective effect, whereas the animals fed the vitamin E-supplemented diets had an increased survival time compared to controls at irradiation doses of 650–850 rads (Figure 1). As part of a larger investigation of radioprotective mechanisms of vitamins, the liver microsomal drug-metabolizing system was studied because radiation-induced suppression of drug metabolism has been observed in rats.

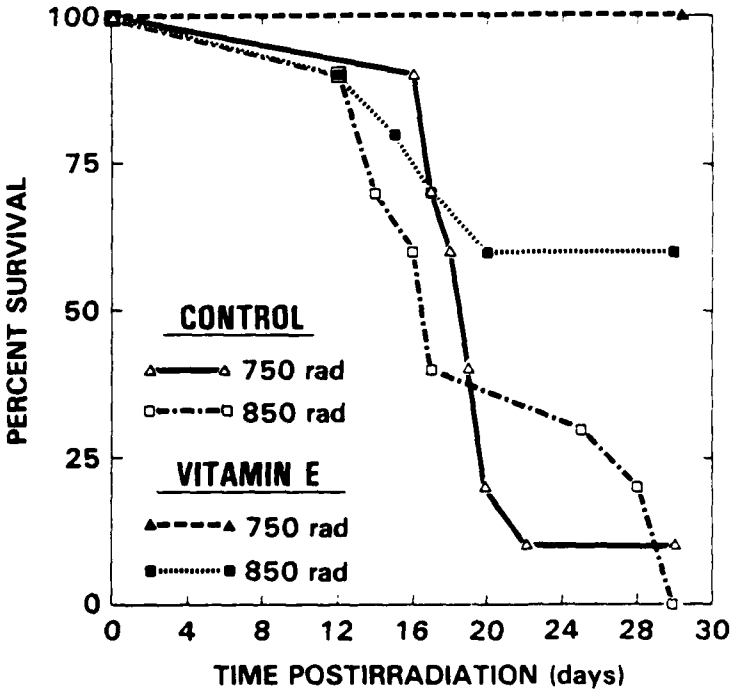

Figure 1. Postirradiation survival of male CD2F1 mice (10 per group) fed control diet or vitamin E-supplemented diet

Animals fed vitamin E- or vitamin B_6-supplemented diets were sacrificed on day 1, day 3, or day 7 after exposure to radiation (700 rads). Cytochrome b_5, cytochrome P-450, and p-nitroanisole demethylase activity were measured in liver microsomes. Demethylase activity did not differ among the groups. Furthermore, no significant difference was seen in cytochrome b_5 and cytochrome P-450 between the animals given vitamin B_6 and the control irradiated animals, whereas the vitamin E-supplemented animals had depressed levels of these parameters compared to the irradiated control animals.

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BOLOGICAL MARKERS: URINE STUDIES

Principal Investigators: J. F. Weiss, C. E. Elhardt, M. A. Donlon, and G. N. Catravas
Technical Assistance: V. L. Cummings, C. M. Morris, W. W. Wolfe, and J. E. Egan

A number of physiological substances have been proposed as biochemical indicators of radiation damage. Some of these markers (urinary creatinine, sialic acid, seromucoid, histamine, zinc, and taurine) are being assessed as biologic dosimeters.

Potential biochemical indicators of radiation damage in the urine of adult, male, Sprague-Dawley rats are under evaluation (1). Rats were fed a controlled amount of diet daily and water ad libitum, and urine was collected from them in metabolic cages. Individual rats (10 per group) were studied for 5 days preirradiation for determination of basal values and for 7 days after exposure

to 100, 300, 500, 700, or 900 rads cobalt-60 irradiation. Sham-irradiated rats were also studied. Urine volume postirradiation followed definite patterns that depended on radiation dose (Figure 1).

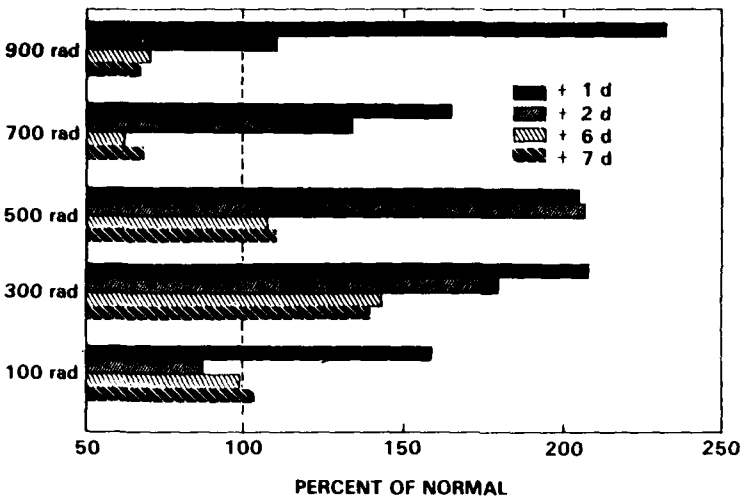

Figure 1. Urine volume of male Sprague-Dawley rats postirradiation

The urine volume increased for 1 or more days, depending on the dose, and decreased after the initial increases at 700 and 900 rads. Automated methods were used to estimate the creatinine, the protein-bound sialic acid after sulfosalicylic acid precipitation, and the sialic acid assayed directly in urine as thiobarbituric acid-reactive material. Creatinine and sialic acid values at 24 hours were significantly different at 1 day after radiation exposure, compared to basal values. The ratio of protein-bound sialic acid to creatinine was higher 1 day after radiation exposure at 500 rads and greater, and may be related to increased serum levels of glycoproteins or protein-bound carbohydrates that are known to increase after radiation exposure.

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BIOLOGICAL MARKERS: SERUM PROTEINS

Principal Investigators: C. E. Elhardt, J. F. Weiss, and A. J. Jacobs, *AFRRI*
Collaborator: P. B. Chretien, *National Institutes of Health*
Technical Assistance: J. C. Jeng and C. M. Morris, *AFRRI*

Serum glycoproteins are significant consequences of radiation damage, trauma, and other disease states, whereas other serum proteins are depressed due to trauma. Evidence exists that several serum glycoproteins that are affected by exposure to radiation have immunoregulatory properties. Assay of these proteins may provide information on immune status (1).

Current studies emphasize the isolation of the protein markers myoglobin and α_2HS-glycoprotein, and also the development of assays for these proteins as well as fibronectin and α_1-acid glycoprotein. The effect of α_2HS-glycoprotein and α_1-acid glycoprotein on macrophage cytotoxicity and chemotaxis was studied.

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BIOLOGICAL MARKERS: VOLATILE HYDROCARBONS

Principal Investigators: J. F. Weiss, C. F. Ehardt, and G. N. Catravas
Technical Assistance: K. M. Hartley

Studies have continued on the possibility of using volatile hydrocarbons expired after radiation exposure as indicators of free radical damage in vivo.

Procedures for measuring the evolution of hydrocarbons in vitro were established. The feasibility of using the generation of volatile hydrocarbons as an index of radiation-induced lipid peroxidation was demonstrated (1).

Techniques are being developed for determining in vivo the evolution of hydrocarbons in order to directly measure lipid peroxidation due to radiation.

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RADIOPROTECTION BY IMMUNOMODULATORS

Principal Investigators: J. F. Weiss, V. Srinivasan, and K. F. McCarthy, *AFRRI*
Collaborator: M. A. Chirigos, *National Institutes of Health*
Technical Assistance: J. C. Jeng, C. L. Harding, and P. W. Jones, *AFRRI*

The potential utility of immune adjuvants (biologic response modifiers) as radioprotective agents is being investigated. Currently, various aspects of radioprotection by the immunomodulators levamisole, thymosin, azimexon, and pyran are being studied. Conversely, the immunoprotective and immunostimulatory properties of known sulfhydryl-containing radioprotective agents such as WR-2721 are being investigated. In this regard, it was determined that the antioxidant and radioprotective properties of levamisole are probably due to its conversion to a sulfhydryl derivative and to modulation of oxidative processes (1).

Studies on azimexon have concentrated on its effects on progenitor cells. Results suggest that this drug may have immunorestorative properties even when administered postirradiation (2). Azimexon, a cyanazaridine immunomodulator, has been shown to increase the survival of X-irradiated mice in our own and other laboratories. Evidence exists that azimexon treatment after radiation exposure helps to reverse radiation-induced leukopenia. Similarly, the toxicity of cyclophosphamide can be reduced in mice by azimexon treatment.

In considering the immunoprotection shown by azimexon, we studied its effect on hematopoietic progenitor cells. Female B6D2F1 mice were injected intraperitoneally with azimexon (25 mg/kg). At various days, spleen and bone marrow cells were cultured for determination of granulocyte-macrophage colony-forming cells (GM-CFC) and monocyte-macrophage colony-forming cells (MM-CFC). Colonies counted 7 days after initial plating were considered to be derived from GM-CFC's, whereas colonies arising after 25 days of incubation were scored as progeny of MM-CFC's.

In both bone marrow and spleen, azimexon treatment resulted in an initial slight depression in number of GM-CFC's, followed by a period of steadily increasing numbers and a significant increase at the 7- to 9-day period. Bone marrow MM-CFC's did not increase until 7 days after treatment. Splenic MM-CFC's were the least affected by azimexon treatment, suggesting different MM-CFC subpopulations (Figure 1). The

increase in granulocytic and monocytic colony-forming cells may play a role in azimexon protection against radiation-induced and drug-induced toxicity.

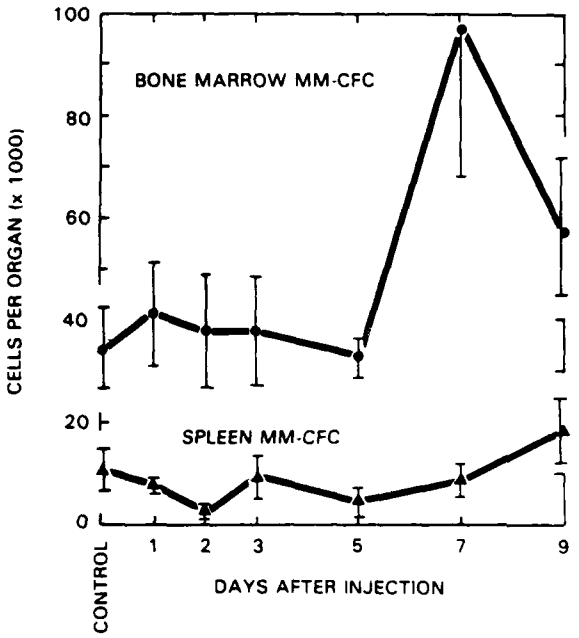

Figure 1. Effect of azimexon (25 mg/kg) on mouse monocyte-macrophage colony-forming cells

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MEASUREMENT OF URINARY HISTAMINE: DEVELOPMENT OF METHODOLOGY AND NORMAL VALUES

Principal Investigators: G. Myers, *National Institute of Allergy and Infectious Diseases, National Institutes of Health*
M. A. Donlon, *AFRRI*
Collaborator: M. A. Kahner, *NIAID, NIH*
Technical Assistance E. A. Helgeson, *AFRRI*

In humans, histamine is released from tissue mast cells as a result of midlethal and lethal doses of radiation. The determination of histamine levels in the blood is complicated by the presence of active plasma histaminases. Approximately 1 percent of the histamine that is injected subcutaneously or intravenously is excreted into the urine. This small portion of histamine, cleared by the kidney and excreted intact into the urine, might allow the analysis of fluctuations in plasma histamine. The measurement of histamine in the urine has several advantages: stability, accessibility, and the opportunity for retrospective analysis.

A method for measuring urine histamine was developed based on cation-exchange chromatography, organic solvent extraction, o-phthalaldehyde condensation, and measurement of fluorescence. A portion of each sample was digested with diamine oxidase in order to determine authentic histamine in the urine.

Normal urinary histamine levels of 13 + 8 ng/ml, 14 + 9 µg/24 hours, or 14 + 12 ng/mg creatinine/ml were found. Ordinarily, males and females excrete equivalent concentrations; spot, short-timed, or 24-hour collections provide equivalent results; and histamine in frozen urine is stable for 6 months or longer. Two patients with systemic mastocytosis and two with idiopathic anaphylaxis were found to have elevated levels of urine histamine.

These results suggested that monitoring the levels of urine histamine may be useful in assessing the conditions in which histamine plays a role. We shall investigate histaminuria in experimental animals following radiation, and assess the effectiveness of several antiallergic and radioprotectant agents on the excretion of histamine.

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EXPERIMENTAL HEMATOLOGY DEPARTMENT

Exposure to ionizing radiation doses of 100-200 rads will damage or destroy bone marrow cells, resulting in the reduction of or the cessation in production of granulocytes, macrophages, and platelets, which are the first and major defense against infectious bacteria and their toxins. With increased radiation doses above 200 rads, these infections result in fatalities.

Infections and fatalities can be decreased by procedures that protect bone marrow cells from these effects, or that enhance their endogenous production postirradiation, or that temporarily supply functional granulocytes until the radiation-damaged bone marrow recovers. In addition, successful treatment is promoted by means that would prevent the invasion of intestinal gram-negative bacteria into other tissues and organs of irradiated persons or would at least reduce the concentration of those bacteria. Successful treatment would increase the percentage of survivors exposed to minimum lethal doses of ionizing radiation. The effect would be to reduce a 10%-20% lethal dose to one that is sublethal.

Studies are conducted to (a) develop therapy for damage from exposures to higher radiation doses that do not completely destroy bone marrow stem cells and (b) develop replacement of bone marrow. In addition, one important project deals with the complex effects of multiple injuries, and another project deals with the possible late effects in survivors of low doses of radiation.

The Departmental program is divided into six project groups, each researching a specific area.

PROJECT GROUP 1: Stem Cell Physiology and Enhancement of White Cell Production Postirradiation

These projects are intended to elucidate (a) mechanisms of normal stem cell renewal and differentiation, (b) interaction of the humoral substances released by functional white blood cells (lymphocytes and/or macrophages) in loci of inflammation and infection, and (c) the primitive precursor

cells for increased production of adult functional cells. Of particular interest is the task of learning how to manipulate the radiation-injured precursor system by molecular engineering in order to enhance the production of white blood cells to fight against invading bacteria and their toxins. Significant progress has been made in determining the humoral and cellular interactions, the proliferation capabilities of normal and postirradiation stem and precursor cells, and the relative biological effectiveness of neutron radiation of these cells.

PROJECT GROUP 2: Studies of Origin and Prevention of Infection Postirradiation

These projects deal with experimental designs to discover the possible routes of bacterial invasion postirradiation, the means of preventing this invasion, and the means of increasing a radiation-injured organism's defense against the bacteria and their toxins. Studies were conducted to determine the relative effectiveness of bacterial lipopolysaccharide (endotoxin) and yeast cell wall preparations (glucan) on stimulation of the hematopoietic system and nonspecific resistance to infection. Mutant mice, resistant to endotoxin and extremely sensitive to gram-negative bacteria, were introduced to help elucidate the mechanisms involved in infectious disease.

PROJECT GROUP 3: Combined Injury

Military analysts have estimated that, in a future atomic war, more than 70 percent of the casualties will suffer certain injuries in addition to those caused by ionizing radiation. The greater percentage of those injuries will be wounds or burns. German and Russian studies using mice or dogs indicate that the presence of open wounds after irradiation will increase the number of fatalities whereas the immediate suturing of open wounds will not. Unfortunately, because of septic conditions, surgeons usually postpone the suturing of wounds in military field conditions. Since the hematopoietic system is involved in the healing of wounds, it is important to study that system's functional status in irradiated animals. Studies to date point out that the incidence of survival is affected by the size of wounds and by the timing of trauma in relation to exposure to radiation. It has been shown that wound trauma before irradiation provokes an earlier and greater increase in clonogenic cells than seen in

irradiated mice. This increased survival after lethal irradiation has been correlated with the proliferation of endogenous pluripotent stem cells.

PROJECT GROUP 4: Physiological Assessment of Fresh and Cryopreserved Granulocytes and Macrophages Used for Post-irradiation Transfusion

Bone marrow exposed to radiation doses of 350-500 rads still has the capability of recovering if the animal or human does not die from infection. The best treatment is the infusion of compatible granulocytes. Methodology for the isolation of granulocytes by counterflow centrifugation-elutriation (CCE) was continued and improved, and the separation of whole bone marrow cells was initiated. We demonstrated that the principles of CCE can be extended to an enlarged separation chamber for the isolation of therapeutic numbers of highly purified granulocytes as well as nucleated bone marrow cells for animal-model transfusion studies. We also showed that CCE-isolated canine granulocytes display differential *in vitro* and *in vivo* efficacy in physiological function as a result of the procedures of dual leukapheresis, CCE isolation, storage, and freezing.

PROJECT GROUP 5: Transplantation of Bone Marrow Cells Into Lethally Irradiated Animals

Once radiation completely destroys the bone marrow, no endogenous recovery is possible. In such a case, the transplantation of bone marrow between genetically identical persons is the only means of treatment and recovery. However, genetically identical cells usually are not available (with the exception of those from identical twins), and the transplantation of incompatible bone marrow results in death. In preparation for establishing a large-animal transplantation model, the methodology associated with counterflow centrifugation-elutriation has been applied to the separation of whole bone marrow suspensions into various cell fractions based on a density gradient. Cellular profiles were obtained from bone marrow suspensions of mouse, dog, monkey, and human. These fractions were each assayed for the presence of clonogenic progenitor cells with repopulating ability.

PROJECT GROUP 6: Late Effects of Ionizing Radiation

In recent years, the possibility has become apparent that military personnel exposed to very low doses of radiation may show an increase of degenerative diseases years later. To obtain greater insight into this phenomenon, studies in this project group were initiated along two major lines of investigation: (a) the effect of low-dose ionizing radiation on pregnant mice and on fetal and neonatal hemopoietic tissue, and (b) the effect of low-dose radiation on long-term *in vitro* cultures of hemopoietic stem cells.

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THYMIC INHIBITION OF MYELOPOIETIC PROLIFERATION

Principal Investigator: D. F. Gruber

The marked radiosensitivity of thymic tissue is a known phenomenon. Some investigators have noted morphological thymic alterations after radiation doses as small as 50 rads (1). This may be due to the fact that ontogenetically a preponderance of thymic tissue is lymphoidal, and lymphocytes are supposed to be radiosensitive. Of interest to us as well as the cells themselves are the factors elaborated by the cells in culture.

We chose to investigate media conditioned by thymus tissue because (a) thymus tissue is uniquely susceptible to radiation, (b) its metabolites affect hematopoiesis, and (c) the thymus has an obvious role in immunoregulation. Some of the functional assays conducted on the conditioned medium from cultures of murine "nonlymphoid" adherent thymus cells show the thymus to be a source of a potent myelopoietic inhibitor substance(s).

Examination of the inhibitor(s) in the soft-agar clonogenic assay (Table 1) shows that it abrogates both the 10-day granulocyte-macrophage colony-forming cell (GM-CFC) and the 25-day monocyte-macrophage colony-forming cell (M-CFC). The inhibition was most significant when the thymus-conditioned medium (TCM) was present at culture initiation. But when TCM was

Table 1. Inhibition and Reversibility of Thymus-Conditioned Medium (TCM) Demonstrated by GM-CFC* and M-CFC†

Treatment	GM-CFC (x̄ ± SEM)	% of Control	M-CFC (x̄ ± SEM)	% of Control
L-cell medium	18.8 ± 0.9	100	10.2 ± 0.8	100
L-cell medium, TCM‡	0	0	0	0
L-cell medium, TCM, one wash§	14.5 ± 0.6	77	15.2 ± 0.9	78
L-cell medium, TCM, two washes	17.4 ± 0.5	93	18.2 ± 0.9	95

* 10-day granulocyte-macrophage colony (> 50 cells) count per 2.2×10^4 live nucleated cells plated

† 25-day monocyte-macrophage colony (> 50 cells) count per 2.2×10^4 live nucleated cells plated

‡ Thymus-conditioned medium (2.5% by volume L-cell medium)

§ Washed in medium and centrifuged at 250 x g for 10 minutes

added at a time after soft-agar initiation (day 6), significant clonogenic inhibition was seen (Table 2).

Table 2. GM-CFC* and M-CFC† Response to Graded Doses of Thymus-Conditioned Medium (TCM) Added 6 Days After Initiation by L-Cell Medium‡

Volume % TCM Added	GM-CFC ($\bar{x}$ ± SEM)	% of Control	M-CFC ($\bar{x}$ ± SEM)	% of Control
0	17.8 ± 1.4	100	18.2 ± 0.9	100
2.5	12.8 ± 1.7	72	13.5 ± 0.6	74
5.0	10.8 ± 1.5	60	4.3 ± 0.5	23
12.5	4.8 ± 0.6	27	4.5 ± 0.5	25
25.0	4.7 ± 0.9	26	4.2 ± 0.3	23

* See footnote 1 in Table 1.

† See footnote 2 in Table 1.

‡ L-cell-conditioned medium, 200 µl

We have characterized the inhibitor(s) as being potent on the basis that volume ratios of inhibitor to L-cell colony-stimulating factor as low as 1 part to 40 parts will give near total inhibition of both GM-CFC and M-CFC clonogenic growth (Table 3). Physiochemically, the inhibitor is dialyzable, has a molecular weight of less than 1000, is not significantly cytotoxic (Table 4), and its effects are reversible with washing (Table 1).

The possibility that this factor is a lymphokine cannot be ruled out completely because a decreasing lymphocyte presence was seen through day 12 of culture (Cytospin morphological analysis). In all likelihood, however, the factor(s) is most probably due to members of an adherent "nonlymphoid" cell population. It appears that the in vitro cells elaborating the inhibitor do so in the absence of either antigenic or mitogenic stimulation, in contrast to other thymic lymphokines (i.e., CIF/PIF) identified in the literature.

Table 3. Graded Dose Inhibition of GM-CFC* and M-CFC† by Thymus-Conditioned Medium (TCM)

Treatment	GM-CFC (x̄ ± SEM)	M-CFC (x̄ ± SEM)
L-cell medium‡	17.8 ± 1.4	18.2 ± 0.9
L-cell medium + 2.5 vol % TCM	1.0 ± 0	1.0 ± 0
L-cell medium + 5.0 vol % TCM	0	0
L-cell medium + 10.0 vol % TCM	0	0
L-cell medium + 25.0 vol % TCM	0	0

* See footnote 1 in Table 1.

† See footnote 2 in Table 1.

‡ L-cell-conditioned medium, 200 µl/plate

Table 4. Percent Viability of Murine Bone Marrow Grown in Graded Quantities of Thymus-Conditioned Medium (TCM)

Volume % TCM Added to Control Medium	Percent Viability* Tested at:			
	6 hr	24 hr	30 hr	48 hr
0	99	97	95	95
2	87	86	85	83
5	83	82	80	80
10	82	82	80	79
25	83	81	81	79

* Determined by hemocytometer and trypan blue exclusion

A number of investigators have implied that thymic humoral factors are elaborated by morphologically appearing epithelial cells. On the basis of histologic and morphologic criteria, it appears that epitheloid cells represent a small fraction (25%) of the adherent thymus cell culture population. The majority (80%) of

the cells appear to be of macrophagic lineage, based on their demonstration of positive esterase staining and ingestion of IgG-coated bovine red blood cells. Quantitatively, these morphological percentages agree with the descriptions of thymic microenvironmental cells reported by Jordan and Crouse (2) and Jordan et al. (3).

In itself, the suppression of myelocytopoiesis may not be significant, but critically important is the awareness of any and all channels of immunocompromise in an organism debilitated by radiation. On the basis of what has been reported here, it appears that TCM must be considered in cases of immunosuppression.

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DEVELOPMENT OF A NEW TECHNIQUE FOR CYTOKINETIC ANALYSIS: BrdUrd/NEAR-UV-CONDITIONED LETHALITY

Principal Investigator: M. P. Hagan
Collaborators: M. M. Elkind and T. J. MacVittie
Technical Assistance: D. P. Dodgen and R. T. Brandenburg

Present techniques for measuring growth rate, cell-cycle time, or the fraction of cells cycling are based on in vitro tests or in vivo suicide. These techniques preclude in vivo measurements prolonged over the lifetime of the cell. Therefore, most of the significant information regarding a cell's lineage remains hidden. This problem could be partially solved with the application of a DNA-labeling technique that could (a) label DNA over a defined period, and (b) represent a target for the formation of a lethal lesion. From previous work it appears that the use of a BrdUrd label for DNA followed by an exposure to near-ultraviolet light (near-UV) fulfills these requirements (1).

The effectiveness of this technique was first tested in a series of in vitro experiments with V79 Chinese hamster cells. The current experiments in this series identify the S-phase of the cell cycle as a time during which cells partially labeled with BrdUrd can still survive large doses of near-UV radiation (1).

Cells labeled with BrdUrd in the same S-phase as the near-UV irradiation demonstrate the ability to repair sublethal damage. This fact is reflected in Figure 1, in

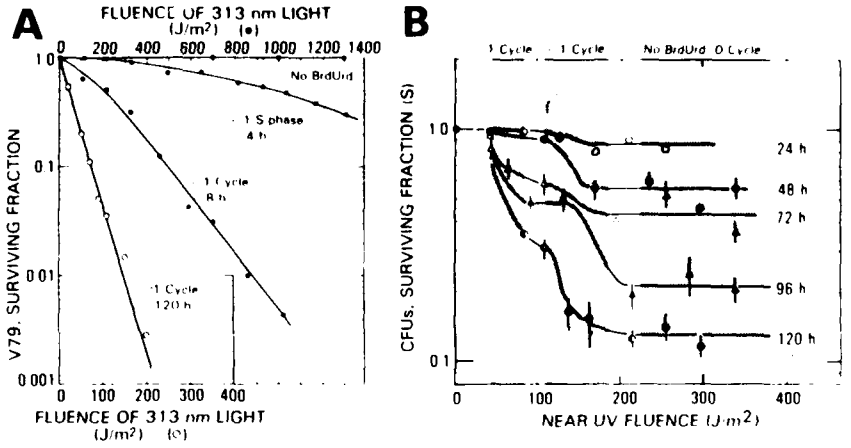

Figure 1. Ultraviolet light irradiation survival curves after incorporation of BrdUrd into DNA. A: V79 cells were labeled *in vitro* for time shown on figure. Label consisted of a solution of 1 x 10^{-5} M BrdUrd, 1 x 10^{-6} M FdUrd, and alpha-modified Eagle's minimal essentials medium. B: Murine bone marrow cells were labeled *in vivo* via a subcutaneous infusion of 75 mM BrdUrd. Error bars, when larger than data symbols, represent ± 1 SE.

the shoulder on the survival curve for V79 Chinese hamster cells. In contrast, cells labeled for one complete generation or more do not repair sublethal damage. This repair of sublethal damage in the S-phase is also independent of caffeine, a drug that sensitizes cells to BrdUrd and near-UV after this initial S-phase. It is then a conclusion of this work that cells labeled with BrdUrd for less than one generation can be easily distinguished from those labeled over longer periods (1).

This work was also extended to the labeling in vivo of murine hemopoietic stem cells. Low replacement levels of BrdUrd for thymidine were achieved through the use of indwelling osmotic minipumps containing sterile aqueous solutions of BrdUrd. The near-UV survival curves produced by this technique permitted the immediate assay of three distinct cell populations: those cycling more than once, those cycling for a fraction of the S-phase, and those not cycling. As shown in Figure 1, the bone marrow stem cell sensitivities to BrdUrd/near-UV are directly relatable to the in vitro analog studies. The kinetic analysis of these data revealed for the first time the rate at which bone marrow-derived stem cells pass from a noncycling state to a cycling state (2).

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GROWTH OF CANINE BONE MARROW AND PERIPHERAL BLOOD MONONUCLEAR CELLS IN *IN VIVO* PLASMA CLOT DIFFUSION CHAMBERS IN MICE: CYTOPOIETIC ACTIVITY, GM-CFC CONTENT, AND PHYSICAL SEPARATION

Principal Investigators: T. J. MacVittie and K. F. McCarthy
Technical Assistance: J. L. Atkinson, E. G. McCarthy, and R. T. Brandenburg

Normal and regenerating canine bone marrow and normal peripheral blood mononuclear cells (PBMC) were cultured in vivo in liquid diffusion chambers (LDC) and in plasma clot diffusion chambers (PCDC) implanted intraperitoneally within sublethally irradiated mice.

The cytopoietic activities (ratio of nucleated cells harvested per diffusion chamber at day 7 of culture to the nucleated cell input) of bone marrow and PBMC were significantly greater when grown within the plasma clot rather than liquid diffusion chamber cultures. Bone marrow and PBMC grown in plasma clot cultures had respective cytopoietic activities of 2.8 and 1.3, while bone marrow and PBMC grown in liquid cultures each had a respective cytopoietic activity of 1.2 and 0.7 (Table 1). Linear relationships were observed between the number of nucleated cells innoculated and the number of cells harvested after 6-8 days of culture.

Table 1. Cytopoietic Activity of Canine BMC and PBMC Grown in Liquid and Plasma Clot DC's*

	Cytopoietic Activity	
	Liquid	Plasma clot
Normal BMC	1.2 ± 0.09	2.8 ± 0.21
Regenerating BMC	—	6.6 ± 1.1
Normal PBMC	0.7 ± 0.09	1.3 ± 0.16

*Values are slopes (± SEM) of regression lines calculated for relationship between the number of nucleated cells inoculated into DCs and the nucleated cells harvested per DC at day 7 of culture. (—) indicates the value was not calculated.

BMC, bone marrow cells

DC, diffusion chamber

Morphologically, both types of cultures resulted in predominant growth of granulocytes, monocytes, and macrophages. The cellular growth curves of bone marrow in LDC and PCDC cultures were markedly different. Total nucleated cells per diffusion chamber in liquid cultures decreased after implantation to a minimum value at day 4, and then rose to control levels by days 7-8. In contrast, the cellularity of PCDC cultures increased within 24 hours and continued to rise, reaching plateau levels (two- to fourfold input) at days 6-8 of culture (Figure 1).

Granulocyte-macrophage colony-forming cells (GM-CFC) measured by in vitro agar culture could not be detected in suspensions derived from liquid diffusion chamber cultures, whereas GM-CFC derived from PCDC cell suspensions remained at concentrations equivalent to input levels over 48 hours and then decreased slowly through day 7 of culture (Figure 2).

Separation of canine density-"cut" bone marrow cells by velocity sedimentation indicated that the population of cells responsible for growth within PCDC is heterogeneous and that a subpopulation of these cells could be separated from GM-CFC (Figure 3). This PCDC population sedimented more slowly (3.7 mm/hour) than the GM-CFC (5.5 mm/hour). A second and more rapidly sedimenting peak was also observed for cells with cytopoietic activities and PCDC at 6.4 mm/hour. Significant overlap was seen with the range of GM-CFC.

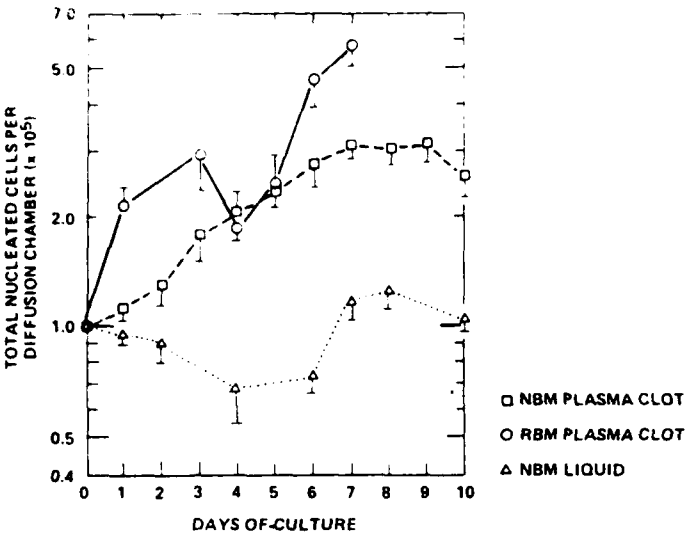

Figure 1. Increase in total nucleated cells per diffusion chamber versus number of days in *in vivo* culture. Diffusion chambers were inoculated with normal canine bone marrow (NBM) in liquid culture and plasma clot culture and regenerating bone marrow (RBM) in plasma clot culture. Mean data points (+ SEM) are from ten (NBM) and four (RBM) replicate experiments, one canine per experiment. Six diffusion chambers were used per data point per experiment.

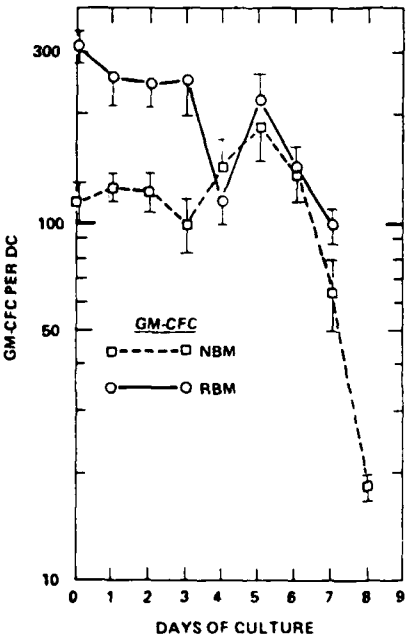

Figure 2. Nucleated cells harvested per diffusion chamber at day 7 of culture versus number of density-cut nucleated bone marrow cells inoculated per diffusion chamber. Values are means of individual experiments, eight chambers per point. Regenerating and normal bone marrows (RBM, NBM) were inoculated in plasma clot and liquid cell suspensions.

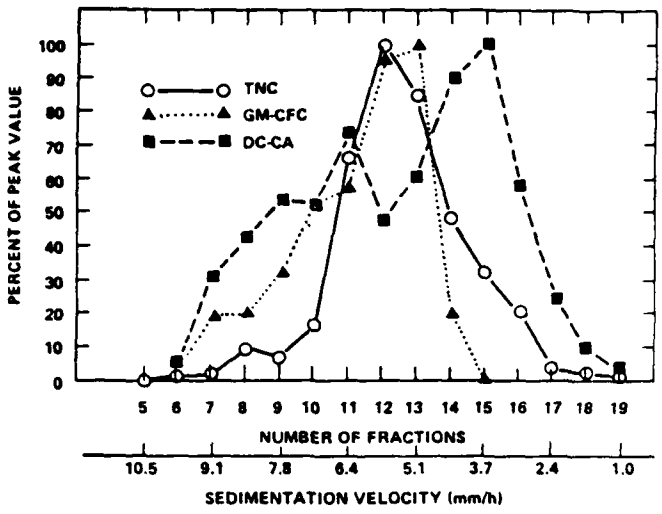

Figure 3. Velocity sedimentation profile of density-cut normal canine bone marrow cells, granulocyte-macrophage colony-forming cells (GM-CFC), and cells responsible for diffusion chamber cytopoietic activity (DC-CA). Illustrated are the mean number of total nucleated cells (TNC), GM-CFC, and DC-CA per fraction of three experiments, expressed as percentage of the peak value versus fraction number and sedimentation rate.

The data showed a significant advantage in growth of canine bone marrow cells and PBMC in plasma clot diffusion chambers over liquid diffusion chambers, in terms of cellularity and growth of GM-CFC. Velocity sedimentation results indicated a separation of a population of cells responsible for growth in PCDC from the in vitro GM-CFC population. This may implicate a parent-progeny relationship between these cells similar to that recently described with human bone marrow cells in fibrin clot diffusion chambers.

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EFFECTS OF LOW-DOSE TOTAL-BODY IRRADIATION ON CANINE BONE MARROW FUNCTION AND CANINE LYMPHOMA

Principal Investigators: D. E. Cowall, T. J. MacVittie, G. A. Parker, and S. R. Weinberg
Technical Assistance: J. L. Atkinson and R. T. Brandenburg

Low-dose total-body irradiation, 150 rads given in 10 fractions over 5 weeks, is a useful treatment modality for favorable-prognosis lymphomas. Little is known, however, about the effects of this regimen on normal bone marrow.

Six healthy beagle dogs and five dogs of various breeds with lymphoma were treated with total-body irradiation. Three of the five lymphomatous dogs achieved remissions of limited duration. No changes in the hemograms or in bone marrow cellularity (as assessed by needle marrow biopsies) could be detected during or after treatment. Bone marrow progenitor cells were studied weekly during treatment and for 4 weeks thereafter using in vitro growth assays for granulocyte-macrophage colony-forming cells (GM-CFC) and monocyte-macrophage colony-forming cells (M-CFC). These studies demonstrated significant reductions ($p < 0.001$) of granulocyte and macrophage progenitor cells with subsequent recovery toward normal preirradiation and sham-irradiation values (Table 1).

Table 1. Canine Bone Marrow GM-CFC* and M-CFC During and After Fractionated, Low-Dose Total-Body Irradiation

Time (weeks)	Cumulative dose (rad)	Normal irradiated		Normal sham irradiated		Lymphomatous
		GM-CFC	M-CFC	GM-CFC	M-CFC	GM-CFC
0	0	$84_6 \pm 6.5$	$110_3 \pm 6.7$	74	113	$65_3 \pm 18.6$
1	30	$48_6 \pm 5.6$	$36_3 \pm 15.6$	63	83	58_1
2	60	$23_3 \pm 9.9$	$34_3 \pm 10.1$	83	95	$29_3 \pm 13.3$
3	90	$26_3 \pm 10.1$	49_2	64	101	$43_3 \pm 27.1$
4	120	11 ± 4.6	$6_3 \pm 3.1$	101	73	$23_3 \pm 10.0$
5	150	$17_6 \pm 7.2$	$49_2 \pm 17.8$	76	85	$12_3 \pm 3.1$
6		$22_3 \pm 11.4$	N.D.	91	74	N.D.
7		$40_3 \pm 5.3$	82_2	111	122	$43_3 \pm 7.2$
8		$46_6 \pm 7.2$	$50_3 \pm 2.4$	71	82	54_1
9		$66_3 \pm 5.3$	64_2	76	81	41_1

* GM-CFC = granulocyte-macrophage colony forming cell; M-CFC = macrophage colony-forming cell. Both are expressed as mean number of colonies 10^5 nucleated cells. Subscript = number of separate experiments, one dog per experiment. N.D. = not determined.

Two other dogs were injected with sublethal doses of Salmonella typhosa endotoxin 2 weeks after completion of the irradiation regimen. Their bone marrow GM-CFC responses were dramatically blunted compared to nonirradiated controls, whereas their peripheral leukocyte responses and serum colony-stimulating activity levels were comparable to those of nonirradiated controls (Table 2).

Table 2. Canine GM-CFC* Response to Endotoxin 2 Weeks After Low-Dose Total-Body Irradiation

Percent of pre-endotoxin GM-CFC values					
	Hours after endotoxin				
	0	24	48	72	96
Irradiated dog 1	100	96	39	85	18
Irradiated dog 2	100	87	94	178	90
Nonirradiated dogs†	100	96	380	350	250

* GM-CFC granulocyte-macrophage progenitor cell in culture.

† Average values for three normal, nonirradiated dogs.

These studies suggest that total-body irradiation may induce bone marrow injury that may be clinically significant if patients so treated are further stressed by infections or myelosuppressive drugs.

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RADIOSENSITIVITY OF EXOGENOUS AND ENDOGENOUS SPLEEN COLONY UNITS AFTER FISSION NEUTRONS AND ^{60}Co GAMMA RAYS

Principal Investigators: R. M. Vigneulle, S. J. Baum, W. S. Bice, and K. P. Ferlic
Technical Assistance: R. T. Brandenburg

A radiobiological program using fission neutrons is in the planning phase at AFRRI in response to needs to expand our technological data base for relative biological effectiveness (RBE) using animal model systems. The present study compares some radiobiological parameters of the hematopoietic stem cells using different techniques and radiation of different qualities in order to establish baseline radiobiological values using either modified fission neutrons from the AFRRI TRIGA Mark-F reactor or ^{60}Co gamma rays.

The modified fission neutrons from the AFRRI TRIGA reactor have a mean energy of approximately 0.7 MeV, a neutron-to-gamma ratio of 32 to 1, and about 3% of total dose contribution due to gamma ray contamination. Groups of mice are rotated vertically as pairs in aluminum tubes along an isodose contour within a 2-inch-thick lead cave open only on the side that faces the reactor. This physical setup minimizes the contribution of gamma dose due to room return.

The radiosensitivity (D_0) of hematopoietic stem cells was estimated, using endogenous spleen colony-forming units (E-CFU) and exogenous spleen colony-forming units (CFU-s) derived from bone marrow and spleen after exposure to ^{60}Co gamma and fission neutrons. The CFU-s were derived from bone marrow or spleen of groups of mice that 24 hours previously had been exposed bilaterally to either (a) 50, 100, 200, 300, and 400 rads of ^{60}Co gamma at 40 rads/minute, using the AFRRI ^{60}Co gamma source, or (b) 50, 100, 150, and 200 rads fission neutrons at 40 rads/minute calculated free in air. The endogenous colonies were measured on spleens of mice fixed in bouins 8 days after exposure to either (a) 400, 460, 518, 575, 632, 690, 748, and 805 rads ^{60}Co gamma or (b) 175, 200, 225, 250, 275, 300, and 325 rads fission neutrons.

Linear regression fits of the data transformed to logs were performed, and the reciprocal slopes were calculated and compared for determination of the relative biological effectiveness (RBE) (Table 1). The RBE (dose of ^{60}Co gamma to the dose of fission neutrons) was calculated (a) at CFU-s surviving fraction equal to 0.1 and (b) using the ratio of reciprocal slopes for endogenous colonies. The RBE was found to range from 2 to 3, although the reciprocal slopes (D_o) were different with each method of measurement.

Table 1. Radiosensitivity and Relative Biological Effectiveness of Committed Stem Cells as Measured by Exogenous and Endogenous Spleen Colonies

	D_o (rad) ± 1 S.E.			
	γ	n	(RBE) γ/n	Method of calculation
Exogenous bone marrow	56 ± 6	29 ± 4	2.3	(S/S_o = 0.1)
Spleen	65 ± 13	23 ± 5	2.9	(S/S_o = 0.1)
Endogenous spleen	165 ± 10	72 ± 6	2.2	(Slope ratio)

In conclusion, although the radiosensitivities determined for hematopoietic stem cells differ and depend on the method of estimation, the RBE that was calculated is not very different. The differences in the radiosensitivities reflect changes in the populations of hematopoietic stem cells measured by each technique.

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ORIGIN, FUNCTION, AND RADIOSENSITIVITY OF CENTRAL NERVOUS SYSTEM MACROPHAGES

Principal Investigators: W. J. Flor and V. M. Hartwig
Technical Assistance: W. L. Sickel

Although mature macrophages themselves are generally considered to be relatively radioresistant cells, evidence exists that the antigen-"processing" step in their function in the immune system can be significantly altered by radiation, thus compromising the irradiated organism's ability to combat infection. Also, the relative radiosensitivity of hematopoietic progenitor and stem cells (from which monocytes and ultimately macrophages are derived) indicate that the macrophage repopulation of an irradiated organism would be significantly diminished. It is believed that the intraventricular phagocytes represent a model system to determine the origin, kinetics, and function of the brain macrophage subpopulation, its relationship to the mononuclear phagocyte system, its sensitivity to radiation, and its response to factors that enhance its production postirradiation.

Normal brain macrophage populations were surveyed by light microscopy, transmission electron microscopy, and scanning electron microscopy in several strains of mice, rats, and guinea pigs. Emphasis was on macrophages within the ventricular system. Macrophage origin was studied by use of ultrastructural and physiological cell markers and by sequential study with the various microscopy systems. Macrophage function was assessed by use of bacteria and by latex or carbon particles administered intraventricularly. Radiosensitivity was determined by gamma irradiation of animals at several doses and rates related to those that affect macrophage subpopulations of other organs, followed by assessment of the presence, stimulation, and function of the irradiated population.

The normal macrophage population of the central nervous system (CNS) of specific pathogen-free rats has been surveyed and found to be greatly reduced from that in conventionally reared rats. This finding indicates that this rat is a good model in which to study the repopulation of the CNS macrophages.

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A LIPOPOLYSACCHARIDE-RESPONSIVE CELL IN C3H/HeJ MICE: THE PERITONEAL EXUDATE-DERIVED MACROPHAGE COLONY-FORMING CELL

Principal Investigators: I. J. MacVittie and S. R. Weinberg
Technical Assistance: L. G. McCarthy and J. L. Atkinson

The C3H/HeJ mouse is a substrain particularly noted for its resistance to the many diverse biologic effects of bacterial lipopolysaccharide (LPS) experienced by many other strains. In addition to decreased lethality and selective in vitro macrophage unresponsiveness, the hematopoietic system of C3H/HeJ is markedly unresponsive to LPS in terms of decreased radiosensitivity, plasma colony-stimulating activity (CSA) levels, splenic stem cells (CFU-s), and granulocyte-macrophage progenitor cells (GM-CFC) than the responsive C3HeB/FeJ strain.

An in vitro colony-forming cell specific for the formation of macrophages has been detected within the murine marrow, extramedullary organs, and tissue spaces. The ubiquitous nature of this macrophage colony-forming cell (M-CFC) and the specific nature of its progeny prompted us to investigate its temporal pattern of induction within the peritoneal cavity of C3H/HeJ mice relative to their counterpart C3HeB/FeJ in response to an intraperitoneal injection of Westphal preparation (LPS-W).

The concentration and total number of M-CFC increased markedly in the peritoneal exudate of both paired strains in response to the intraperitoneal injection of LPS-W (Figure 1), although a marked contrast is noted between the strains during the initial 48 hours. The CH/HeJ M-CFC increased in concentration from 73 per 10^5 to a peak value of 4807 per 10^5 cells at 48 hours, representing a 66-fold increase. This resulted in a 10-fold advantage in the absolute number of M-CFC in the peritoneal exudate cells (PEC) of C3H/HeJ mice over C3Heb/FeJ (Figure 1). This is in spite of the dramatic 176-fold increase in M-CFC concentration in the C3Heb/FeJ over the same 48-hour period. It required an additional 24 hours for the C3Heb/FeJ to catch the C3H/HeJ in relative number of M-CFC and hence approach a comparable absolute number of M-CFC by 72 hours after injection of LPS-W (Figure 1).

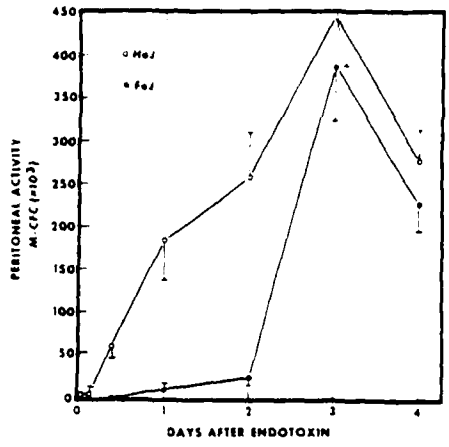

Figure 1. Total macrophage colony-forming cells (M-CFC) detected in peritoneal cavity of C3Heb/FeJ and C3H/HeJ mice following intraperitoneal injection of Westphal preparation, *E. coli*

In view of the high potential for involvement of macrophages in the many diverse biologic reactions to endotoxin and the well-documented unresponsiveness of macrophages as well as granulocyte-macrophage progenitor cells from C3H/HeJ strain mice to these reactions, we measured the response of an in vitro colony-forming cell specific for macrophages to an intraperitoneal injection of LPS-W in both C3HeB/FeJ and C3H/HeJ mice.

Our studies have shown that (a) the C3H/HeJ strain contains a significantly greater number of M-CFC in the hematopoietic organs, peripheral blood, thymus, and peritoneal cavity, and (b) in response to endotoxin, the peritoneal exudate is characterized by a more accelerated rise in content of M-CFC within the initial 48 hours after injection. At 48 hours, the C3H/HeJ PEC contained 257,000 M-CFC versus 25,000 M-CFC for the C3Heb/FeJ. Peak values, however, were comparable at 72 hours in both strains. This is probably the result of a marked rise in migration and proliferation of M-CFC in the C3Heb/FeJ.

Concomitant with the marked rise in absolute values of M-CFC in the C3H/HeJ is a biphasic increase in neutrophils and macrophages. This response confirmed that reported by Sultzer and Goodman (1) and by Moeller et

al. (2). The C3H/HeJ responded more quickly, eliciting an early polymorphonuclear increase within 6 hours, followed by a rapid decrease to negligible numbers within 48 hours. A rapid rise in macrophages ensued. Sultzer and Goodman concluded that the C3H/HeJ is not a low responder in terms of the peritoneal inflammatory response. We have also shown that it is capable of an accelerated response in terms of the peritoneal exudate-derived M-CFC. The control data also indicated a significantly greater number of M-CFC in other organs and tissue spaces also affected by endotoxin. The bone marrow and extramedullary organs and tissue spaces appear to be primed in this respect for a rapid increase in systemic M-CFC.

The diversity of the LPS-induced activities affected by this mutation have recently been shown to include many aspects of the hematopoietic response. The cellular effect is most evident in the splenic tissue where endogenous colony-forming units, exogenous CFU-s, and GM-CFC are all significantly diminished in their characteristic in vivo responses to endotoxin. Recent experiments analyzing the temporal response to endotoxin of the M-CFC in bone marrow, spleen, and peripheral blood of C3H/HeJ mice have shown significant differences from the responses of their earlier progenitors, the CFU-s and GM-CFC (unpublished observations). These various populations of M-CFC (bone marrow, spleen, peripheral blood leukocytes, PEC) are markedly responsive during the initial 48 hours after endotoxin challenge. The exact relationships between the M-CFC, the response of the hematopoietic and mononuclear phagocyte systems to endotoxin, and the defective cellular mechanism in C3H/HeJ mice remain to be determined.

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RESPONSE OF MURINE MACROPHAGE COLONY-FORMING CELLS TO ENDOTOXIN IN C3Heb/FeJ AND C3H/HeJ MICE

Principal Investigators: T. J. MacVittie and S. R. Weinberg
Technical Assistance: E. G. McCarthy and J. L. Atkinson

The inbred mouse strains C3Heb/FeJ and C3H/HeJ differ in response of the monocyte-macrophage colony-forming cell (M-CFC) to bacterial lipopolysaccharide-W (LPS), whereas they show equivalent responses of M-CFC as well as granulocyte-macrophage colony-forming cell (GM-CFC) to agents such as Corynebacterium parvum and Mycobacterium bovis strain Bacillus Calmette-Guérin (Table 1).

Table 1. Alterations in Splenic Cellularity and Content of GM-CFC and M-CFC in C3Heb/FeJ and C3H/HeJ Mice After Injection of *C. parvum* or BCG*

Days		*C. parvum*		*M. bovis*	
		C3Heb/FeJ	C3H/HeJ	C3Heb/FeJ	C3H/HeJ
0	TNC	15.11 ± 1.1	17.23 ± 1.6	10.13 ± 1.3	16.35 ± 1.4
	GM-CFC	2.39 ± 0.2	2.21 ± 0.3	1.71 ± 0.3	1.86 ± 0.3
	M-CFC	38.81 ± 4.1	82.11 ± 6.8	22.86 ± 3.4	75.21 ± 5.8
5	TNC	26.18 ± 2.3	27.31 ± 2.7	25.65 ± 1.9	28.26 ± 2.9
	GM-CFC	75.32 ± 5.8	131.26 ± 10.2	27.35 ± 4.1	39.41 ± 5.5
	M-CFC	301.16 ± 18.1	425.77 ± 23.4	128.17 ± 11.7	683.47 ± 32.6
11	TNC	47.78 ± 4.8	51.30 ± 4.5	52.40 ± 3.1	49.57 ± 2.7
	GM-CFC	83.60 ± 7.4	97.72 ± 8.3	69.90 ± 11.9	94.24 ± 12.2
	M-CFC	410.87 ± 12.3	429.31 ± 23.1	250.06 ± 9.3	1,238.33 ± 47.3

*Mean values ± SEM of 3 replicate experiments. TNC × 10^6, organ values × 10^3

The M-CFC of both strains were assayed in femoral marrow, spleen, and peripheral blood following an intraperitoneal injection of 10 µg LPS. The cellular and humoral responses of the two strains were in marked contrast (Figure 1), with the exception of the rise in circulating levels of leukocyte-derived M-CFC, which increased to the same degree in each strain. M-CFC derived from the marrow of both strains showed significant decreases within 6 hours. However, the M-CFC of the C3Heb/FeJ reached a nadir by 24 hours, followed by a slow rise to control values; the M-CFC of the C3H/HeJ, after a slight rise, dropped and remained at

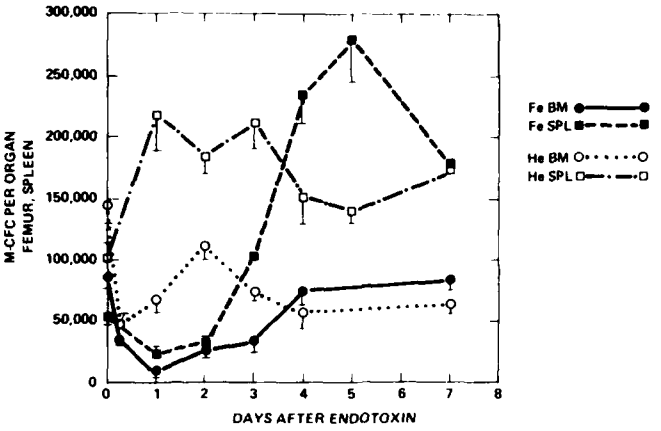

Figure 1. Number of monocyte-macrophage colony-forming cells (M-CFC) per femur and spleen in C3Heb/FeJ and C3H/HeJ mice at various times after intraperitoneal injection of 10 µg of *E. coli* lipopolysaccharide-W (LPS). Values are means (± SEM) of six replicate experiments.

values only 40% of control through 7 days. C3Heb/FeJ spleen-derived M-CFC increased to levels 500% of control within 5 days whereas the C3H/HeJ spleen M-CFC showed an early rise to values 220% of control within 24 hours, remained at this level through 72 hours, and then decreased to values 150% of control through day 7.

Peak values for colony-stimulating activity capable of stimulating M-CFC-derived colonies were observed 3 to 5 hours after LPS in the C3Heb/FeJ mice, whereas plasma levels of colony-stimulating activity did not rise above control in the C3H/HeJ mice.

The data suggested that, in normal LPS-sensitive mice, the M-CFC forms part of the cellular response of the hematopoietic organs to endotoxin, whereas the qualitatively different response observed in the M-CFC of the C3H/HeJ mice may have resulted from the transient mobilization of hemopoietic cells from the bone marrow without the consequent proliferation and differentiaton.

The presence of the M-CFC in many extramedullary organs and tissue spaces suggests that it may play a significant role in nonspecific cell-mediated responses of the mononuclear phagocyte system to microbial infection.

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DOSE-DEPENDENT EFFECTS OF COMMERCIALLY AVAILABLE GLUCAN ON MURINE PLURIPOTENT STEM CELLS AND ON MYELOID AND ERYTHROID PROGENITOR CELLS

Principal Investigators: M. L. Patchen and T. J. MacVittie
Technical Assistance: G. A. Davis and J. L. Atkinson

The immunomodulating agent glucan has been shown to alter the proliferation of pluripotent stem cells (CFU-s) and granulocyte-macrophage colony-forming cells (GM-CFC) in murine bone marrow and spleen (1,2). Because of glucan's ability to modulate hemopoiesis, it has been proposed as a possible agent for enhancing hemopoietic recovery after radiotherapy, chemotherapy, and bone marrow transplantation and for combating infectious complications.

In the past, glucan has been available only through collaboration with a limited number of investigators producing the substance. The result has been that the magnitude of glucan-induced hemopoietic responses as well as the maximum tolerable glucan dose has varied from source to source. Recently a standard glucan preparation became commercially available. We investigated a wide range of hemopoietic effects produced by six different doses of this glucan preparation.

C3H/HeN mice were intravenously injected with either 0.1, 0.4, 0.8, 1.2, 1.6, or 2.0 mg of glucan. Five days later, total nucleated cellularity, CFU-s, GM-CFC, macrophage colony-forming cells (M-CFC), erythroid burst-forming cells (BFU-e), and colony-forming cells (CFU-e) were assayed in the bone marrow and spleen.

Neither femoral cellularity nor GM-CFC (Figure 1) content was altered by any of the glucan doses tested. By contrast, bone marrow CFU-s (Figure 2) content increased at all glucan doses tested, and CFU-e (Figure 3) and BFU-e (Figure 4) contents decreased with increasing glucan doses. In the spleen, all aspects (Figures 1-3,5) of hemopoiesis (i.e., cellularity, CFU-s, GM-CFC, M-CFC, and CFU-e) increased after glucan administration. The degree of splenic stimulation was directly correlated with the dose of glucan administered.

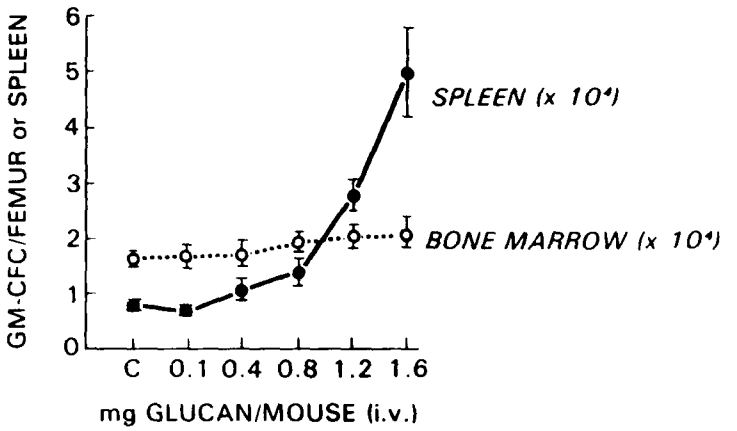

Figure 1. Saline (C) or glucan (0.1, 0.4, 0.8, 1.2, 1.6, or 2.0 mg) was intravenously administered to mice. Five days later, the bone marrow (o----o) and spleen (●—●) were assayed for granulocyte-macrophage hemopoietic progenitor cells (GM-CFC). Each datum point represents the mean ± SEM of values obtained from three individual experiments.

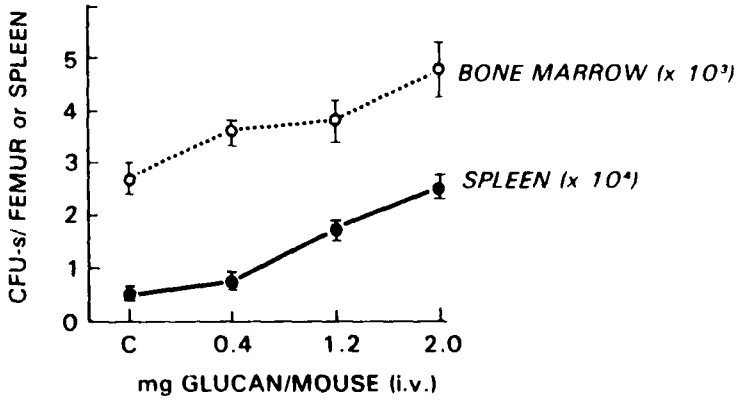

Figure 2. See legend for Figure 1 except: assayed for pluripotent hemopoietic stem cells (CFU-s).

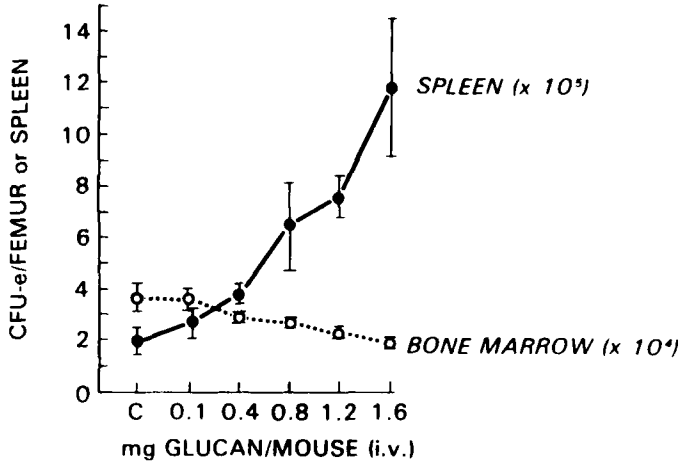

Figure 3. See legend for Figure 1 except: assayed for erythroid colony-forming hemopoietic progenitor cells (CFU-e).

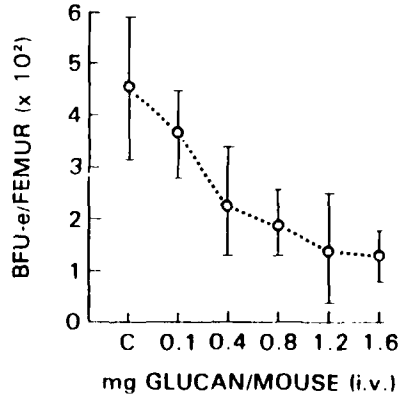

Figure 4. See legend for Figure 1 except: assayed for erythroid burst-forming hemopoietic progenitor cells (BFU-e).

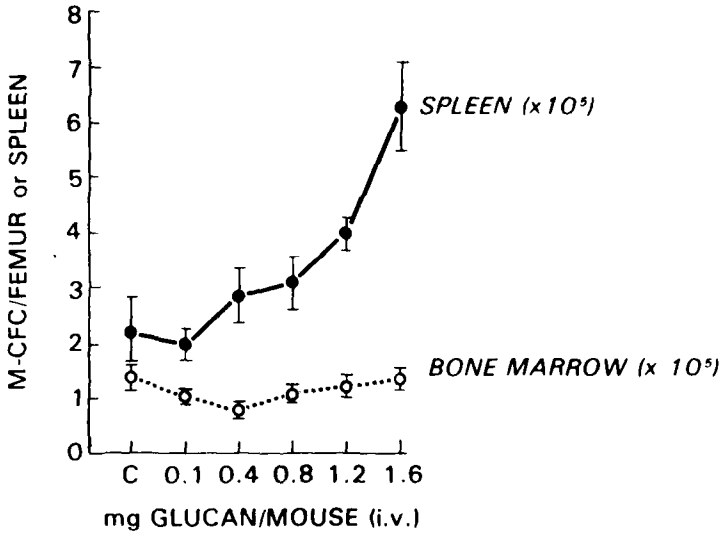

Figure 5. See legend for Figure 1 except: assayed for pure macrophage hemopoietic progenitor cells (M-CFC).

These studies indicate that, at the proper dose, the glucan assayed in these studies can be used to overall effectively enhance CFU-s, GM-CFC, M-CFC, and erythroid proliferation in mice. Specifically, a 1.6-to 2.0-mg dose of this glucan can stimulate approximately a twofold increase in the number of bone marrow CFU-s and fivefold, sixfold, threefold, and sixfold increases in the numbers of splenic CFU-s, GM-CFC, M-CFC, and CFU-e, respectively.

It is hoped that the availability of consistent glucan preparations will allow research to expand in the area of discovering the mechanisms of glucan's action on altering the various parameters of hemopoietic proliferation, and in the area of applying glucan to aid in hemopoietic repopulation following hemopoietic injury. Currently, we are investigating glucan's ability to assist in enhancing hemopoietic recovery following radiation-induced hemopoietic injury.

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SURVIVAL AND ENDOGENOUS SPLEEN COLONIES OF IRRADIATED MICE AFTER SKIN WOUNDING AND HYDROXYUREA TREATMENT

Principal Investigators: G. D. Ledney, H. M. Gelston, Jr., S. R. Weinberg, and E. D. Exum

Survival (1,2) from radiation that is induced by wound trauma may be related to increased mitosis in hematopoietic cells. This hypothesis was tested by two experimental protocols using hydroxyurea. The resulting data support the idea that survival from lethal irradiation induced by prior skin wounding is related to the proliferation of endogenous colony-forming units-spleen (E-CFU-s).

Wounding and irradiation were performed on 14- to 16-week-old B6CBF1 mice. Methyoxyflurane-anesthetized mice were wounded in the anterior dorsal skin fold and the underlying panniculus carnosus muscle with a steel punch repeatedly cleaned by immersing in 70% ethanol. The wound was 2.0-2.5 cm^2, which was 4% of the total skin surface. Wounding was done 24 hours before exposure to ^{60}Co, between 10:00 a.m. and 2:00 p.m. The wounds were left untreated and open to the environment. Irradiated, nonwounded control mice were anesthetized before irradiation. All animals were exposed to whole-body radiation at a rate of 40 rads/minute from bilateral ^{60}Co sources.

In the first series of experiments, 30-day survival studies were done with mice given 900 rads. The cell cycle-dependent drug hydroxyurea (HU) (Sigma Chemical, St. Louis, MO) was dissolved in sterile distilled water (50 mg/ml) and injected into mice intraperitoneally (1 mg/g body weight) at selected times before or after irradiation. In the second experimental series, 8-day E-CFU-s were counted in mice after wounding and 700-rad treatments.

In preliminary studies we determined that (a) no 8-day E-CFU-s were detectable in mice given only 900 rads and (b) wounding before 900 rads resulted in only 1-2 E-CFU-s per spleen. While that number of E-CFU-s was sufficient to promote survival from 900 rads, we used 700 rads to better quantify E-CFU-s responses in combined-injury animals treated with HU.

Results. In the first series of experiments, seven treatment groups were established. The 30-day survival fractions of three of these groups are shown in Figure 1. HU injected into either nonwounded-irradiated or

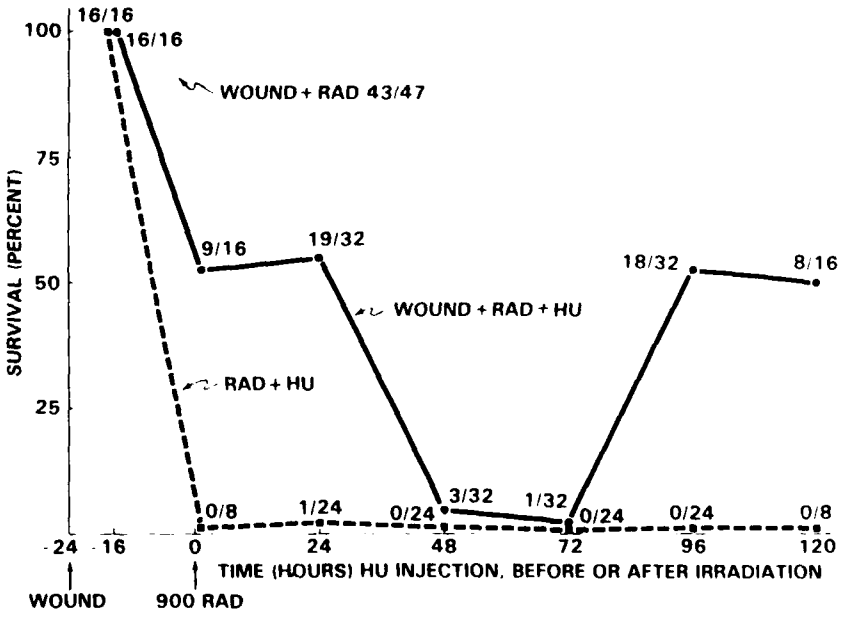

Figure 1. Thirty-day survival fractions of skin wounded-irradiated mice treated with hydroxyurea (HU). Mice were wounded 24 hours before irradiation with 900 rads ^{60}Co. HU (1 mg/g) was injected into mice at 16 hours before irradiation, or 10 minutes after irradiation, or at 24-hour intervals for a 5-day period after irradiation. Fraction at each point is number of survivors/number of treated mice. ●——●, wound + rads + HU. ■---■, rads + HU. Survival fraction of control wounded and irradiated mice was 43/47. All mice (n = 48) given only 900 rads died, and all mice (n = 16) only wounded survived.

wounded-irradiated mice 16 hours before irradiation resulted in 100% survival. However, injection of HU into nonwounded-irradiated mice shortly after exposure or at daily intervals for 5 days thereafter was associated with nearly 100% mortality. In mice wounded before irradiation, 40%-95% of the animals died after injection with HU. These mortality rates are compared to the 10% mortality found in wounded-irradiated mice not given HU. Survival (after radiation) induced by skin-wound trauma was maximally blocked by treatment with HU on either day 2 or day 3 postirradiation. In the remaining four groups of control mice, all animals given 900 rads died, whereas all mice lived that were wounded only, or injected with HU only, or injected with HU after wounding.

The second set of experiments also contained seven experimental groups. Figure 2 shows the number of E-CFU-s detected in four groups of animals. These are mouse groups given (a) 700 rads alone, (b) wounding + 700 rads, (c) HU + 700 rads, or (d) wounding + 700 rads + injection with HU. In irradiated mice, wounding

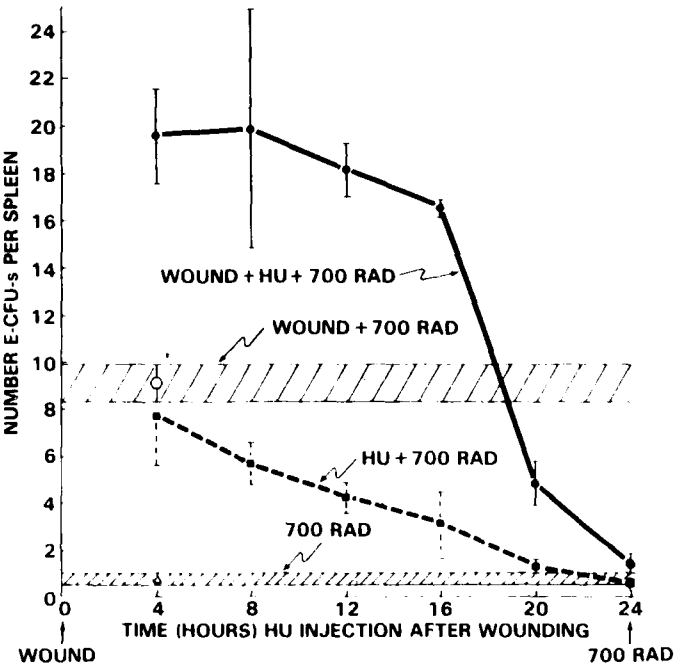

Figure 2. Endogenous colony-forming units-spleen (E-CFU-s) in wounded mice given hydroxyurea (HU) before irradiation with 700 rads ^{60}Co. Mice were wounded at time 0, and HU (1 mg/g) was injected into animal groups at 4-hour intervals, beginning at 4 hours after wounding and ending at 10 minutes before irradiation. ●—●, wound + HU + 700 rads. ■---■, HU + 700 rads. ○, wound + 700 rads. △, 700 rads only.

resulted in nine nodules/spleen whereas less than one nodule/spleen was found in irradiated-nonwounded animals. Treatment of wounded animals with HU at selected times before irradiation caused an increase in splenic nodules almost double the increase in wounded-irradiated mice without the drug. When wounded mice were injected at either 4 hours or 10 minutes before irradiation, the numbers of colonies were approximately the same numbers found in irradiated-nonwounded control animals. No E-CFU-s were found in mice either (a) wounded alone, (b) injected with HU, or (c) injected with HU after wounding.

The data presented in this report reflect on a cell compartment that is necessary for survival from the combined trauma of radiation injury and wounding, and that is depleted by the appropriately timed treatment with HU.

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REGULATION OF AN ENZYME THAT METABOLIZES ACTIVE OXYGEN

Principal Investigator: B. H. Gray
Technical Assistance: R. W. Stull

It has been hypothesized that irradiation leads to the leakage of endotoxin from the mammalian gut, causing shock. The administration of endotoxin results in the production of superoxide and peroxide in mouse lungs, and exogenous superoxide dismutase (SOD) at an optimum concentration reduces mortality due to endotoxin (1). However, it was also found that endotoxin lethality is increased in mice following injection of four times the optimal SOD concentration (2). This enhanced mortality may result from a requirement for superoxide radical by one endotoxin-inducible enzyme found in mouse lung: indoleamine-2,3-dioxygenase (IDO) (3). This enzyme reduces the serotonin levels in lungs using superoxide radical as the second substrate.

Figure 1 shows the drop in SOD and the concomitant induction of IDO in mouse lung (3,4). SOD appears to be under metabolic control to achieve regulation of the superoxide radical concentration. SOD activity is decreased, resulting in an increase in the concentration of superoxide radical upon serotonin release in the lungs. The regulation and control of SOD is important because the enzyme is a known factor in cell protection upon irradiation. The regulation of SOD is currently under study.

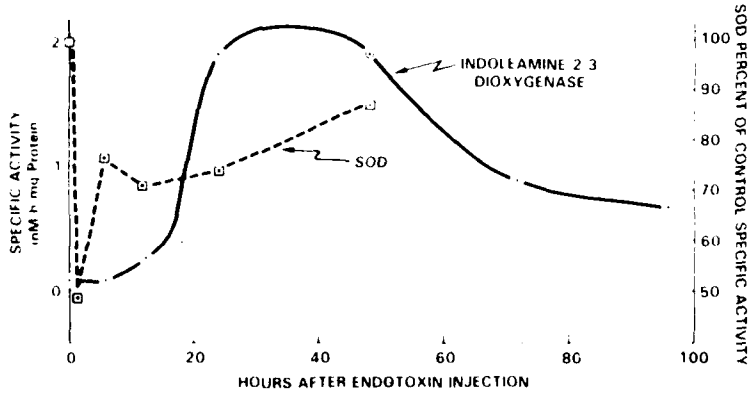

Figure 1. Specific activity of exogenous superoxide dismutase (SOD) and indoleamine-2,3-dioxygenase (IDO) in mouse lung after injection of endotoxin

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POSTIRRADIATION *IN VIVO* ANALYSIS OF PRESERVED GRANULOCYTES

Principal Investigators: J. F. Jemionek, T. J. Contreras, and W. H. Baker
Technical Assistance: D. Walden

The in vivo functional capability of fresh and liquid stored granulocytes was evaluated by transfusing therapeutic levels of granulocytes into neutropenic canine models and measuring the chemotaxis of the cells into implanted skin chambers. Storage temperatures (6°C and 20°C) and storage times (0 and 24 hours) were evaluated for granulocyte-rich leukapheresis concentrates and for elutriation-purified granulocytes. The therapeutic quantities of granulocytes were obtained by continuous-flow centrifugation leukapheresis (granulocyte-rich concentrate) and then purified by using counterflow centrifugation-elutriation with the enlarged rotor system (28.8 ml) built at AFRRI.

The data indicate that the in vivo chemotactic functions were the same for granulocytes transfused at time 0 hour from either granulocyte-rich leukapheresis concentrates or elutriation-purified granulocytes (Table 1). However, the elutriation-purified granulocytes,

Table 1. PMNL Migration Into Skin Chambers for Control Animals and Animals Transfused With Fresh PMNL From CFCL Concentrates or CCE-Purified Cell Preparations*

	Positive Control[+]	Negative Control[±]	CCE-Purified PMNL[§]	CFCL Concentrates[§]
Mean	49.3	0.10	8.21	10.41
± SEM	15.6	0.03	3.43	4.58
	(n = 4)	(n = 4)	(n = 4)	(n = 4)

* Data expressed as number of white blood cells x 10^6 migrating into skin chamber. Means underlined are not significant at 5% level ($p > 0.05$).

[+] Positive control. Migration into chambers of canines not treated with cyclophosphamide and not given a PMNL transfusion.

[±] Negative control. Migration into chambers of canines rendered neutropenic by cyclophosphamide but not given a PMNL transfusion.

[§] Experimental tests. Migration into chambers of canines rendered neutropenic by cyclophosphamide and given a PMNL transfusion of either CCE-isolated or CFCL concentrates within 3 hours of final collection.

PMNL, granulocytes
CFCL, continuous-flow centrifugation leukapheresis
CCE, counterflow centrifugation-elutriation

when stored for 24 hours at either 6°C or 20°C, exhibited a severely impaired in vivo chemotaxis (Table 2). In contrast, the granulocytes from the leukapheresis concentrate, when stored at 20°C, showed an improved in vivo chemotaxis compared to the 0-hour data. Storage of these later granulocytes at 6°C was less beneficial to the observed activity at time 0 hour (1).

Table 2. PMNL Migration Into Skin Chambers for CCE-Isolated PMNL or CFCL Concentrates Held for 18-24 Hours at 6°C or 20°C Before Transfusion*

	Cells Held for 18-24 Hours at 6°C as:		Cells Held for 18-24 Hours at 20°C as:		
	CCE-Purified PMNL	CFCL Concentrate	CCE-Purified PMNL	Dextran-Separated WBC	CFCL Concentrate
Mean	0.98	5.41	0.45	3.04	14.56
± SEM	0.31	2.37	0.17	2.37	0.98
	(n = 5)	(n = 3)	(n = 3)	(n = 2)	(n = 5)

* Data expressed as number of white blood cells x 10^6 migrating into skin chamber after transfusion into cyclophosphamide-induced neutropenic canine. Means underlined by same number of lines (single or double) are not significantly different at 5% level ($p > 0.05$).

Abbreviations same as in Table 1

In vitro data were contradictory, since it had been previously observed that stored elutriation-purified granulocytes retain the functions of chemotaxis, phagocytosis, and bactericidal activity. Thus, the use of in vitro analysis may be limited in its ability to indicate in vivo function as a measure of success in granulocyte preservation studies.

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IN VITRO ANALYSIS WITH EMPHASIS ON ULTRASTRUCTURE OF CRYOPRESERVED LEUKOCYTES FOR POSTIRRADIATION TREATMENT

Principal Investigators: V. M. Hartwig and W. J. Flor

Membranes and intracellular structures of leukocytes (granulocytes, lymphocytes, and monocytes) of canines and humans were evaluated to determine a correlation between the ultrastructure and function. These observations were used to establish criteria to formulate improved methods for the cryopreservation of granulocytes used in postirradiation therapy.

Canine and human leukocytes were isolated from peripheral blood by centrifugation leukapheresis followed by counterflow centrifugation-elutriation. Cell viability and integrity were evaluated by measuring the following parameters: mean cell volume and size distribution (with the H-4 Coulter Counter System), cell viability (with ethidium bromide assay), chemotactic ability (with the modified Boyden chamber technique), phagocytic capability [using latex beads, fc receptor(s), chicken red blood cells, tantalum, and scanning electron microscopy methods], and analysis of ultrastructural changes (using scanning and transmission electron microscopy) (1).

Techniques have been established for monitoring cellular membrane and/or ultrastructural changes by electron microscopy in correlation to cryopreservation methods, cell culture, and radiation effects. Methodology was established to convert subjective observations of scanning and transmission electron microscopy to quantitative terms suitable for direct and comparative statistical analysis.

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ISOLATION OF PROGENITOR CELLS BY COUNTERFLOW CENTRIFUGATION-ELUTRIATION

Primary Investigators: J. F. Jemionek, W. H. Baker, and T. J. MacVittie
Technical Assistance: D. Walden

Fractionation of mouse, canine, monkey, and human bone marrow cells by counterflow centrifugation-elutriation (CCE) was used for the isolation of granulocyte-macrophage colony-forming cell (GM-CFC) activity (1). The cell separation was achieved by varying the viscosity of the elutriation medium from a specific gravity of 1.0123 to 1.0394 using bovine serum albumin, 2-14 gm% (weight per volume) in phosphate-buffered saline. A linear density gradient was used for elutriation.

Pretreatment of the bone marrow cells with Ficoll-Hypaque resulted in a different elutriation profile for both cellular distribution and GM-CFC activity than did cells that were nontreated. This divergence of results favored the elutriation of nontreated bone marrow cells.

The bone marrow cells of the canine, monkey, and human elutriated characteristically into two different peaks of nucleated cells (Figure 1). The first peak was elutriated before the density gradient was started, and the second peak was elutriated by the higher density of the medium. However, in vitro culture activity (GM-CFC) of the collected fractions resulted in a species variance.

For the canine, the GM-CFC activity was associated with each of the two nucleated cell peaks. The activity from the monkey was detected primarily in the second nucleated cell peak. The GM-CFC activity for the human bone marrow was more complex, displaying three peaks of activity: one associated with the first peak of nucleated cells, and two associated with the later. The profile for the mouse bone marrow was unique, in that most of the nucleated cells were elutriated in the first fraction.

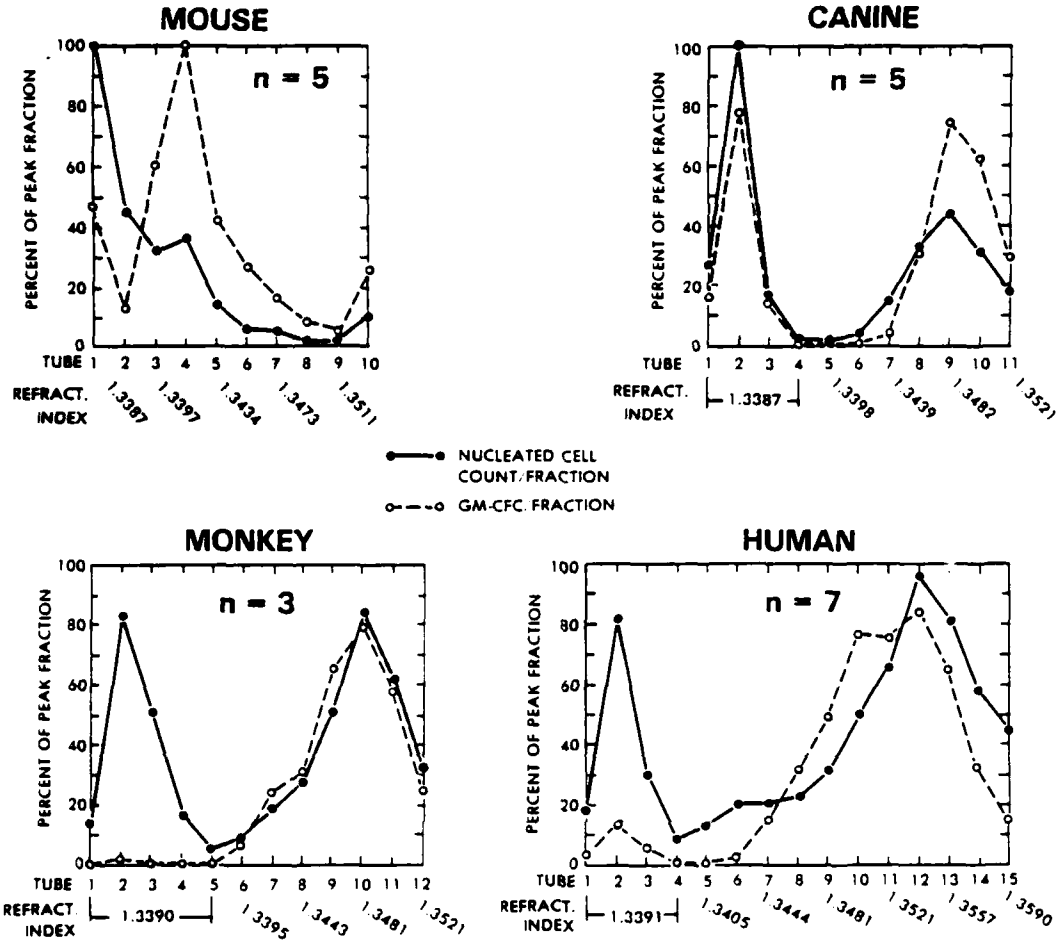

Figure 1. Profile of nucleated cell recovery and granulocyte-macrophage colony-forming cell (GM-CFC) activity in bone marrow from four donor species after separation by counterflow centrifugation-elutriation

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FETAL MURINE HEMOPOIESIS FOLLOWING *IN UTERO* LOW-DOSE IRRADIATION

Principal Investigators: S. R. Weinberg, E. G. McCarthy, and T. J. MacVittie

The influence of in utero exposure to low-dose ionizing radiation on murine hemopoietic embryogenesis was investigated. In vitro assays such as micro plasma-clot cultures and double-layer soft agar cultures served as sensitive biodosimeters to determine erythropoietic and granulopoietic injuries.

Pregnant mice (day 10.5, HA/ICR) were irradiated with 0, 50, 100, 150, 200, or 300 rads. Day-14.5 fetal livers were studied for colony-forming unit-erythroid (CFU-E), burst-forming unit-erythroid (BFU-E), granulocyte-macrophage colony-forming cell (GM-CFC), and macrophage-colony-forming cell (M-CFC) activity.

Fetuses subjected to doses of 200 rads or higher on day 10.5 of gestation responded with a decrease in day-14.5 liver cellularity, reflecting injury to the developing organ and its inability to recover to the nonirradiated values. Difference in responses between erythropoietin(EPO)-dependent and EPO-independent CFU-E strongly suggests the existence of two populations of erythroid progenitor cells with different radiosensitivities. A dose of 200 rads markedly reduced CFU-E recovery, and a dose of 100 rads was sufficient to reduce BFU-E recovery to almost 10% of 0-rad values. Nonirradiated day-14.5 fetal liver had more GM-CFC than did any of the irradiated fetuses, and a dramatically reduced M-CFC recovery occurred with each increase in dose following 150 rads.

Our results showed that (a) fetal liver granulopoiesis is more sensitive to radiation injury than is erythropoiesis, and (b) fetal liver has a greater potential for erythropoiesis recovery.

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EFFECT OF LOW-DOSE IRRADIATION ON PREGNANT MOUSE HEMOPOIESIS

Principal Investigators: S. R. Weinberg, E. G. McCarthy, T. J. MacVittie, and S. J. Baum

The effects of low-dose gamma radiation on hemopoietic progenitor cell compartments of the marrow and spleen of virgin female mice and pregnant mice were studied. Microplasma clot cultures were used to assess burst-forming unit-erythroid (BFU-E) and colony-forming unit-erythroid (CFU-E) activity. Double-layer agar cultures were established to evaluate granulocyte-macrophage colony-forming cell (GM-CFC) and macrophage colony-forming cell (M-CFC).

The apparent shift in maternal erythropoiesis from the bone marrow to the enlarged spleen was reflected by an increase in the numbers of CFU-E and BFU-E per spleen and a concomitant decrease in CFU-E and BFU-E per femur. Although maternal GM-CFC values per femur increased 36%, maternal GM-CFC per spleen increased by 172% compared to virgin values. A greater decrease was seen in M-CFC per spleen than per femur in the pregnant animal when values were compared to the virgin animal.

Total-body irradiation to the day-10.5 pregnant mouse caused a further suppression of day-14.5 medullary erythropoiesis (i.e., decreased CFU-E values) compared to the response of the virgin female mouse. An ability of the maternal spleen to support further compensatory erythropoiesis following increasing doses of radiation was demonstrated. At 4 days after 1.0 Gy exposure, maternal values for GM-CFC per femur or spleen decreased to nonirradiated virgin mice values. M-CFC per maternal femur decreased following 1.5 Gy, but M-CFC per spleen appeared to be unaffected with doses from 0.5 to 2.0 Gy.

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HEMOPOIESIS IN THE SPLENECTOMIZED-PREGNANT MOUSE AFTER LOW-DOSE TOTAL-BODY IRRADIATION

Principal Investigators: S. R. Weinberg and T. J. MacVittie

The effect of splenectomy (SPLX) and total-body irradiation (50-200 rads) on hemopoiesis of virgin mice and pregnant mice was studied, using peripheral blood hemogram values and femoral marrow hemopoietic progenitor cell activity (i.e., CFU_E, BFU_E, and GM-CFC).

The SPLX-maternal red cell counts and hematocrit values were lower than those of SPLX-virgin mice (Figure 1), reflecting the anemia of pregnancy. But the white cell counts of both SPLX-virgin and SPLX-day-14.5 pregnant mice were significantly higher ($p < 0.005$) than normal-virgin mice.

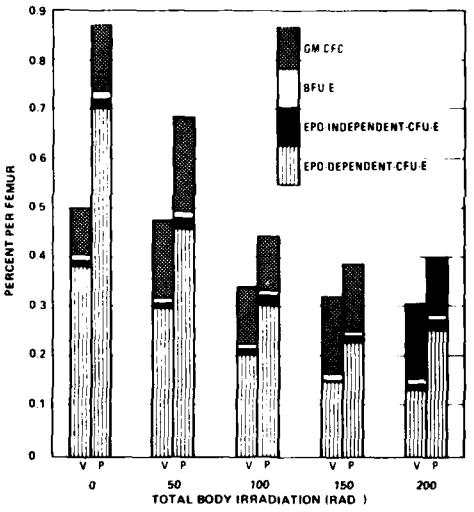

Figure 1. C57BL/6J SPLX-virgin (V) and SPLX-day-14.5-pregnant (P) mean percent values per femur of hemopoietic progenitor cells at 4 days after total-body irradiation: EPO-dependent-CFU_E, EPO-independent-CFU_E, BFU_E, and GM-CFC.

Both nonirradiated and day-4 irradiated SPLX-maternal marrow erythropoietin(EPO)-independent and EPO-dependent CFU_E were higher than the nonirradiated and day-4 irradiated SPLX-virgin values (respectively, for each total-body irradiation dose studied). On the other hand, nonirradiated and day-4 irradiated SPLX-maternal GM-CFC were lower than the nonirradiated and day-4 irradiated SPLX-virgin GM-CFC values.

The data demonstrate the potential of the SPLX-maternal femoral marrow to respond to the stress of low-dose total-body irradiation with effective compensatory erythropoiesis, possibly at the expense of granulopoiesis.

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IN VITRO STUDIES OF LONG-TERM EFFECTS OF RADIATION DAMAGE TO GROWTH AND DIFFERENTIATION OF HEMATOPOIETIC STEM CELLS

Principal Investigator: S. R. Weinberg
Associate Investigators: T. J. MacVittie and V. M. Hartwig

Long-term liquid cultures of fetal and adult mouse and blood-cell-forming tissues of canine were maintained for 10-13 weeks. Cell growth and differentiation of cells were harvested weekly and evaluated. Scanning electron microscopy of these cultures was monitored regularly.

Preliminary studies showed a definite relationship between bone marrow stroma and nonadherent hemopoietic progenitor cells from different strains of irradiated mice (C3H/HeJ and C57BL/Ks) receiving a total-body dose of 60-180 rads of cobalt-60. Radiation injury to the marrow was demonstrated only if the marrow structure was not disturbed when the cultures were initially established. This culture technology has the potential to assess latent effects of in vivo irradiation.

Animal models with virgin and pregnant mice were established to investigate the radiation damage and the recovery of bone marrow following total-body irradiation (50-200 rads cobalt-60). The full compensatory potential of the bone marrow to support hemopoiesis in the irradiated-splenectomized-pregnant mouse was clearly demonstrated. Similar animal models with non-irradiated canines show a similar stress of pregnancy on bone marrow hemopoietic activity. Mouse fetuses exposed in utero to gamma radiation (50-200 rads) demonstrated significant injury to fetal and to neonatal hematopoiesis.

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PHYSIOLOGY DEPARTMENT

Fiscal year 1981 was the first full year of existence for the Physiology Department, a year marked by the growth of the Department's scientific and technical staff.

The Physiology Department is responsible for designing and directing research using various cellular tissue models and whole animal models to study the physiological and biophysical changes that result from exposure to radiation, either alone or in combination with drugs or chemicals. The Department uses an integrated approach that ranges from single cells in isolation, single cells and multiple cells in tissue culture, tissue slices, whole animal recordings, and functional studies in awake, performing animals, to investigate the effect of radiation insult.

The Physiology Department is subdivided into three Divisions. The General Physiology Division conducts research on the physiological responses of the various organ and tissue systems of the mammal (including the endocrine, gastrointestinal, and cardiovascular) to radiation and radiation-related injury. The Neurophysiology Division conducts research on how nerve cells and other components of the nervous system respond physiologically to ionizing radiation. The Radiation Biophysics Division conducts research on the functional and biophysical changes that occur at the membrane and subcellular systems of a number of cell types, in response to radiation insult.

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COUPLING RATIO OF THE SODIUM POTASSIUM PUMP IN THE LOBSTER CARDIAC GANGLION

Principal Investigator: D. R. Livengood

The lobster cardiac ganglion is a unit of nine neurons that control the rhythmic contraction of the lobster's heart. Five of the nine neurons are large enough to be penetrated with as many as three microelectrodes. These cells have been previously shown to have an electrogenic sodium-potassium pump (1).

This electrogenicity is a function of the relative ratio of (a) the sodium ions pumped out of the cell to (b) the potassium ions pumped into the cell. This relative sodium-to-potassium coupling ratio has been the subject of some controversy. Mullins and Brinley (2) suggested that the coupling ratio may be variable in squid axons. But Abercrombie and DeWeer (3) presented evidence indicating that the electrogenic coupling ratio in squid axon seems to be fixed at 3 to 2. Gorman and Marmor (4) found that the electrogenic coupling ratio in Anisodoris neurons is 2 to 1 in a steady-state nonsodium-loaded condition. An electrogenic pump coupling ratio of 2 to 1 was also found in Aplysia neurons (5). These results differ from the findings of Thomas (6) and from Lambert, Kerkut, and Walker (7), who found that the coupling ratio in Helix neurons is 3 to 2. Gorman and Marmor (4) suggested that one explanation for these differences might be the level of sodium loading of the different nerve cells.

The present experiments in lobster cardiac ganglion cells attempted to resolve this issue by using two distinctly different electrophysiological methods to estimate the electrogenic pump coupling ratio in both a sodium-loaded and a nonsodium-loaded condition. Evidence from the two methods is internally consistent. The evidence indicates that in the nerve cells of the lobster cardiac ganglion, the electrogenic pump coupling ratio in both a sodium-loaded and a steady-state nonsodium-loaded condition is 3 to 2 when the ganglion is bathed in artificial seawater containing normal levels of sodium and potassium. These two experimental approaches can be used to evaluate the level of radiation damage to the sodium-potassium pump in most cell types. This pump occurs almost everywhere, and it is vitally important to the steady-state function of most cells.

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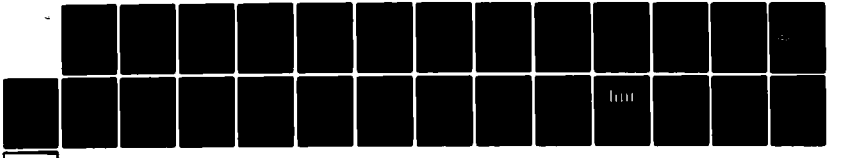

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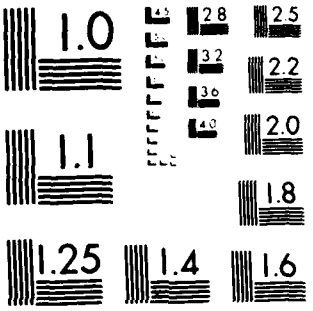

The pump coupling ratio was determined by two different electrophysiological techniques. A graphical analysis, plotting exp(VF/Rt) versus K_o after the pump was blocked by ouabain, was used to determine values for $[K]_i$, P_{Na}/P_K, and the pump coupling ratio gamma. These measurements were made 5 to 8 hours after the cells had been penetrated with microelectrodes. Therefore, these measurements constitute nonsodium-loaded steady-state values. The value obtained for the pump coupling ratio under these conditions was 1.44 + 0.06 (n = 9), or close to three sodiums for two potassiums (Table 1).

Table 1. Values Calculated for $[K]_i$, P_{Na}/P_K Ratio, and Pump Coupling Ratio in Normal Saline

EXPERIMENT NO.	RESTING POTENTIAL	OUABAIN DEPOLARIZATION	CORRELATION COEFFICIENT	$[K]_i$	P_{Na}/P_K	COUPLING RATIO
MN1206	-62	6.3	0.93	305	0.049	1.58
MN3166	-66	4.0	0.77	312	0.037	1.38
MN3116	-63	4.8	0.93	247	0.029	1.59
MN12096	-53	7.0	0.61	276	0.053	1.64
MN9015	-57	8.0	0.94	156	0.015	1.20
MN9245	-62	2.0	0.99	208	0.009	1.54
MN9305	-53	4.0	0.97	322	0.069	1.27
MN9115	-56	2.5	0.94	170	0.016	1.40
MN8155	-61	3.8	0.98	286	0.024	1.32
	-59.2	4.71		253.6	0.033	1.44
	±1.5	±0.67		±20.7	±0.007	±0.05

The second technique used to measure the coupling ratio was to iontophoretically inject sodium ions into the neuron. Neurons were penetrated with three microelectrodes, two of which were filled with 2 M sodium-citrate, and the third with either 2 M potassium-citrate or 3 M potassium chloride. By passing current between the electrodes containing sodium salt, reproducible amounts of sodium could be injected into the cell soma. The injection system was calibrated by injecting sodium-24-citrate into counting vials from representative microelectrodes (calculated transport number = 0.92).

By knowing the sodium load injected into the cells, and by measuring the time-current area produced by sodium activation of the sodium-potassium pump, the coupling ratio was calculated as 1.54 ± 0.05 (n = 19). This is not significantly different from the value obtained by the previous method (Table 2). This value, then, represents a sodium-loaded experimental situation. These results suggest that the pump normally operates with a 3:2 ratio both at rest and under sodium load.

Table 2. Coupling Ratio Values Obtained From Injection Experiments

EXPERIMENT NUMBER	RESTING POTENTIAL (mV)	INPUT RESISTANCE (MΩ)	LOAD INJECTED (picoequiv.)	UNCOUPLED LOAD PUMPED OUT (picoequiv.)	COUPLING RATIO
I 167	-55	1.2	44.2	14.9	1 51
		1.2	24 3	11.2	1 85
		1.2	30.9	12.2	1 65
I 185	-53	2.8	35 0	10.5	1 43
I 186	-50	0.6	26 5	10.9	1 70
		0 6	37 5	19 1	2 04
		0.6	39 7	11 0	1 38
I W23	-60	1.3	23 9	7 3	1 44
		1.4	32 2	9 2	1 40
		1.4	36 8	9 0	1 32
I W16	-55	2.0	38 6	13.5	1 54
		2 0	35 0	11.2	1 47
		2 0	35 0	16 0	1 84
		2 0	58 9	22 5	1 62
I W24	-60	1 3	27 2	8 9	1 49
		0.8	47 3	15 7	1 50
I W76-1	-60	2 0	29 0	10 6	1 58
		2 4	31 3	6 1	1 24
		2 9	36 7	8 7	1 28
	AVG -56 1 ± 1 5				AVG 1 54 ±0 05

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BLOOD FLOW IN CANINE INTESTINE POSTIRRADIATION AS DETERMINED BY HYDROGEN CLEARANCE

Principal Investigators: T. F. Doyle, L. G. Cockerham, and R. B. Trumbo

Radiation-induced early transient incapacitation (ETI) is accompanied by severe systemic hypotension, during which arterial blood pressure often decreases to less than 50 percent of normal. One hemodynamic compensatory mechanism is the increased peripheral resistance caused by vasoconstriction. This vasoconstriction in the small intestine of dogs increases disproportionately during hemorrhagic or endotoxin shock, and intestinal ischemia frequently occurs.

In an attempt to clarify the mechanisms underlying radiation-induced ETI and the gastrointestinal radiation syndrome, the blood flow in the canine intestine was measured by the hydrogen polarographic technique, both before and after exposure to gamma irradiation. Systemic blood pressures, blood gases, and hematocrits were determined simultaneously.

Data obtained from 12 sham-irradiated dogs indicate that 10 K rads of whole-body gamma irradiation produced a 31 percent decrease in systemic mean blood pressure, beginning within 10 minutes postirradiation and lasting for at least 90 minutes. However, the intestinal blood flow did not decrease as anticipated, but instead showed an actual postirradiation increase. This increase in postirradiation intestinal blood flow began within 5 minutes after irradiation and lasted for at least 90 minutes. Also, postirradiation hematocrits were 10.5 percent higher than those obtained before irradiation.

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MEMBRANE PROPERTIES OF MACROPHAGES

Principal Investigator: F. K. Gallin
Collaborator: D. R. Livengood

Studies performed to determine the baseline membrane properties of macrophages have indicated that cultured mouse peritoneal macrophages exhibit voltage-dependent conductances that produce a region of negative slope resistance in their current-voltage relationships (1). As shown in Figure 1, this voltage-dependent conductance is insensitive to tetrodotoxin and cobalt. However, this conductance is blocked by barium ions.

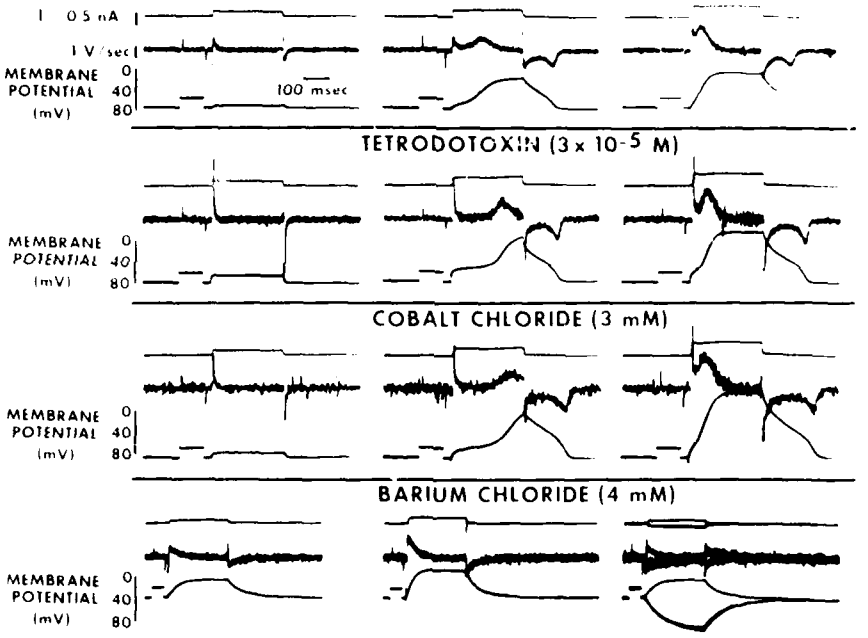

Figure 1. Effect on voltage response of adding tetrodotoxin, cobalt chloride, and barium chloride to injected current pulses of a macrophage exhibiting an S-shaped current-voltage curve. Top line of voltage responses are in normal Hank's. Subsequent lines represent consecutive addition of tetrodotoxin (3×10^{-5} M), cobalt chloride (3 mM), and, finally, barium chloride (4 mM) to bath.

In order to determine if human macrophages exhibit similar properties, cultured human peritoneal macrophages were studied. In these experiments, a voltage-sensitive potassium conductance similar to that described in mouse macrophages was not seen. However, cells that exhibited a calcium spike in response to injected current (2) were recorded. These cells were esterase-positive, but they were not capable of ingesting opsonized red blood cells; therefore, they were not typical macrophages.

These studies are the first to demonstrate that macrophages and macrophagelike cells can exhibit complex rectifying properties similar to other excitable cells.

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EFFECT OF SERUM-FREE MEDIUM ON GROWTH AND DIFFERENTIATION OF SYMPATHETIC NEURONS IN CULTURE

Principal Investigator: J. E. Freschi

Primary cultures of dissociated superior cervical ganglia (SCG) were grown either in serum-containing medium (+FBS) or serum-free defined medium (N_1). The cultures were then compared for several differentiated properties.

Neuronal survivals were similar in the two media. Background cell (especially fibroblast) proliferation was less in N_1. Small intensely fluorescent cells were occasionally seen only in +FBS. Catecholamine fluorescence in neuronal processes and cholinergic synaptic activity (Figure 1) persisted beyond 1 month in culture in both media. Quantitatively, however, the neuritic outgrowth, nerve terminal fluorescence, and synaptic interaction were less in N_1. Muscarinic depolarization and electrical membrane properties, including the presence of at least four voltage-sensitive outward currents, were similar in the two media.

Thus, SCG neurons differentiate in N_1 essentially to the same degree as they do in +FBS.

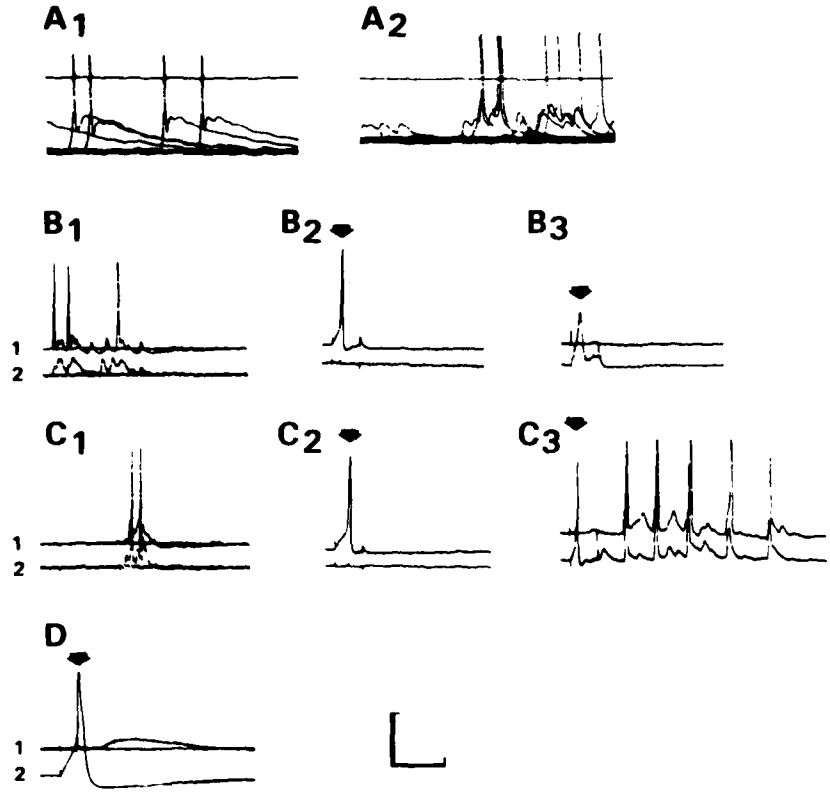

Figure 1. Cholinergic synaptic networks. (A) Spontaneous excitatory synaptic potentials in +FBS cultures. A_1 and A_2 from different cells. (B) Spontaneous synaptic activity occurs synchronously in both neurons (B_1). Evoked action potential (arrow) in either cell 1 (B_2) or cell 2 (B_3) fails to drive other cell. (C) Spontaneous synaptic activity occurs synchronously in both neurons (C_1). Evoked spike (arrow) in cell 1 (C_2) does not drive cell 2, but evoked spike (arrow) in cell 2 (B_3) causes complex synaptic responses in cell 1 and in itself. (D) Driver-follower cell pair in N_1 medium. Action potential in cell 2 evokes monophasic excitatory postsynaptic potentials in cell 1. Vertical: 40 mV for all records. Horizontal: 100 mseconds for B_1, C_1; 40 mseconds for A_1, A_2, B_2, B_3, C_2, C_3; 10 mseconds for D. A-D are from different cells.

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EFFECTS OF IONIZING RADIATION ON THE MONOSYNAPTIC MUSCLE STRETCH REFLEX

Principal Investigator: D. J. Braitman
Technical Assistance: V. A. Kieffer

The electromyogram (EMG) recorded from muscle is a simple technique for determining the functional integrity of the spinal cord. Using this method, we have identified several components pertinent to the EMG complex, namely the M1 and M2 components of the muscle stetch reflex (MSR).

The M1 reflects the monosynaptic activity of the spinal cord when a muscle stretch is elicited (1). The MSR becomes evident when excitation of the primary endings of receptors in the muscle spindles promote an electrical discharge of the motor neurons, specifically in our experiment through the monosynaptic pathway of the ventral spinal roots (C 5-6) and (C 6-8) (2). These axons innervate the biceps brachia and triceps brachia, respectively. M2 activity occurs at approximately 30 mseconds, reflecting a long loop transcortical pathway (3).

In our experimental protocol, rhesus monkeys were required to maintain elbow angle at 90 degrees ($\pm$ 1.5 degrees) against constant extension force. Elbow angle and biceps (EMG) from chronic intramuscular stainless-steel wire electrodes were monitored by computer. If correct angle was maintained for a randomly selected 1- to 2-second period, and if the average absolute value of biceps EMG (sampled at 10 kHz) for the final 0.5 second of the period fell within a preset range, then a stimulus consisting of a 20-msecond pulse of additional extension force occurred at the end of the period. The stimulus transiently extended the elbow (3-4 degrees), which elicited a stretch reflex. M1 was defined as the average absolute value of the EMG occurring 12 to 25.5 mseconds after stimulus onset, minus the prestimulus EMG amplitude. The computer also followed the stimulus-induced changes in elbow angle. Under the control condition, a liquid reward was given 70 mseconds after stimulus onset.

The AFRRI linear accelerator was used to deliver 1200 rads (600 rads/day for 2 consecutive days at an energy of 13.5 MeV to the monkey's cervical spinal cord. The M1, M2, and EMG baseline activities were monitored. At 30 days after exposure, the M1 component of the EMG showed significant increase. No accompanying

change was seen in the M2 or background EMG (Figure 1).

Several monkeys are being trained and will be irradiated for comparison with these data. This experimental model will allow us to determine the underlying effects of ionizing radiation on motorsynaptic function, which result in degradation of combat performance.

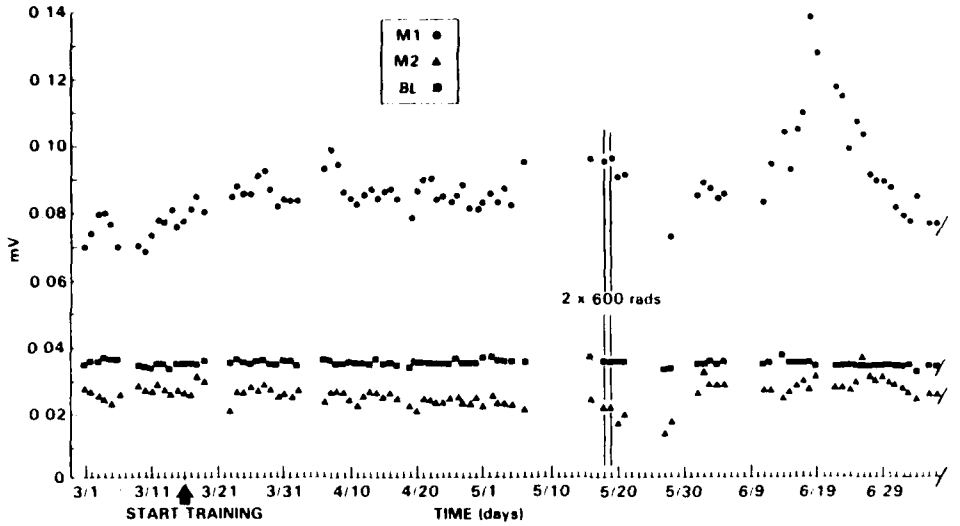

Figure 1. Amplitude of the M1, M2, and baseline EMG responses preirradiation and postirradiation. Each point represents a 24-hour average. A significant increase is observed in the M1 after 1200 rads.

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ANALYSIS OF PROGRESSIVE TOXICITY IN RAT FROM SHALE-DERIVED JP5 VERSUS PETROLEUM-DERIVED JP5

Principal Investigators: W. J. Mehm and C. L. Farmer

Rats were gavaged with either petroleum-derived or shale-derived jet propulsion fuel number 5 (JP5) (24 ml/kg body weight), and sacrificed at selected intervals between 3 hours and 15 days after gavage. Samples of liver tissue and blood were taken and examined for evidence of hepatocellular damage.

Lesions produced from both fuels were found in the periportal region of the hepatic lobule, showing extensive vacuolization (fatty change) and increases in binucleated cells, mitotic figures, and pyknotic nuclei (Figure 1). Visible hepatic lesions appeared 6 hours earlier, and lasted about 1 day longer in the shale-treated rats than in the petroleum-treated rats. This was attributed to a greater degree of hepatotoxicity, caused by the presence of additional chemical constituents in the shale-derived fuel (from extraction and processing requirements). No difference was noted in the nature of the lesions induced by either the shale- or petroleum-drived fuel.

Serum chemistry analyses substantiated the evidence for hepatocellular damage, with elevated levels of serum glutamic pyruvic transaminase (SGPT) and serum glutamic oxaloacetic transaminase (SGOT). Significant differences in these values for the dosed subjects versus the controls occurred as early as 6 hours, and lasted up to 5 days postgavage (Figure 2). Peak levels for both enzymes were seen in animals sacrified 12 hours after gavage.

The work completed thus far has established a comparison of the progressive stages of hepatic damage in rats treated with (a) shale-derived JP5 and (b) petroleum-derived JP5. The occurrence of hepatocellular damage was also substantiated by elevated levels of SGOT and SGPT in the serum.

Now that the nature, sequence, degree, and sites of hepatocellular damage have been observed from ingested JP5, an inhalation study of JP5 (in collaboration with the Behavioral Sciences Department) should be undertaken to histologically determine the dangerous threshold levels of airborne fuel vapor. These data,

combined with the electrophysiological and behavioral parameters determined by BIS, could be valuable in determining unsafe conditions for military personnel working with these fuels.

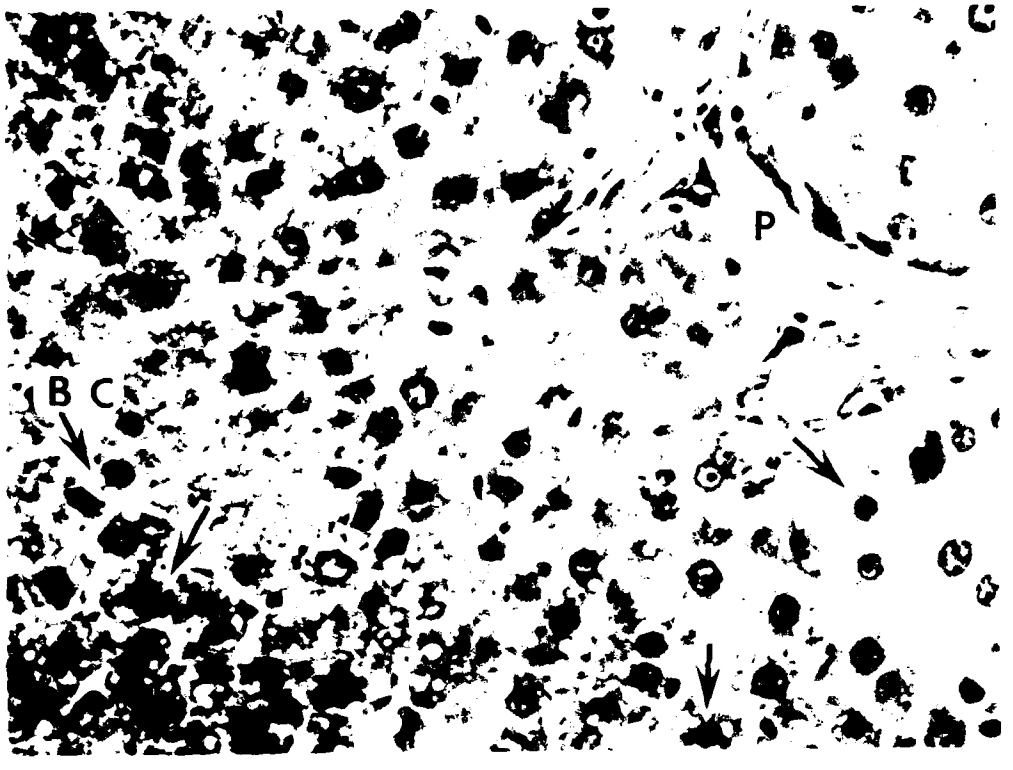

Figure 1. Generalized pattern of maximum periportal vacuolization (fatty change) characteristic of petroleum-derived JP5 induced lesions. Hepatocellular vacuoles (C) can be seen to surround the portal tract (P). Mitotic figures (not readily apparent (arrows)) and a post-mitotic binucleated cell (BC) can be seen. 1 week post-petroleum-gavaged rat, paraffin-embedded, H & E X 250

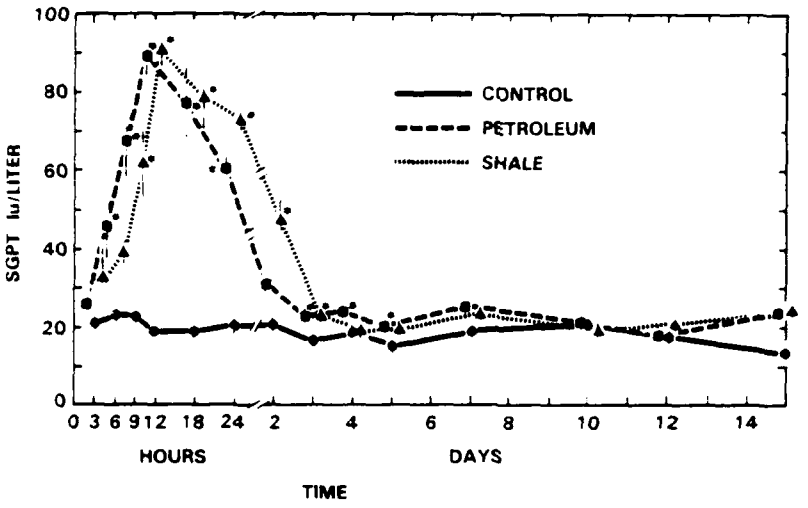

Figure 2. Mean SGPT levels ± standard errors for three treatment groups at times indicated (n = 5). * denotes significant effect of treatment by analysis of variance ($p < 0.05$). Note broken time scale between 24 hours and day 2.

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EFFECTS OF EXCITOTOXIC AGENTS ON THE *IN VITRO* OLFACTORY CORTEX BRAIN SLICE

Principal Investigator: D. J. Braitman
Collaborators: C. R. Auker and S. L. Rubinstein

Ionizing radiation at levels as low as 250 rads causes a significant increase in excitatory electrical activity recorded from rat olfactory cortex (1). We have tested the action of various excitotoxic amino acids on isolated slices of tissue from olfactory cortex in an attempt to model the action of ionizing radiation on this brain tissue.

In an earlier study (2), we demonstrated that kainic acid has a pharmacological profile similar to that of the endogenous transmitter at the terminal synapses of lateral olfactory tract (LOT). Kainic acid is a very

potent excitatory agent; in high concentrations, it is extremely toxic to nerve cells, but it is not a substance naturally found in the brain. The effects of kainic acid on nerve tissue may be similar to those of ionizing irradiation. Recently, Ruck et al. (3) reported that the naturally occurring folic acid derivative, methyltetrahydrofolate (MTHF), is a potent and specific competitor for kainic acid-binding sites in rat cerebellar membranes. In an attempt to identify an endogenous substance that may act at kainic acid receptors, we examined the actions of folic acid and its derivatives formyltetrahydrofolate (FTHF) and MTHF on electrophysiological activity in the olfactory cortex slice.

Thin slices (300-400 μm) of rat olfactory cortex were cut and incubated at 35°C in oxygenated normal Ringer's solution. Each slice was transferred to a recording chamber that was constantly perfused with Ringer's solution. The LOT was stimulated with double shocks of 20-50 volts for 50 μsec via a tungsten bipolar electrode placed across the tract. Paired shocks were separated by 60 mseconds and were administered every 4 seconds. Shock intensities were adjusted to produce field potentials of maximum amplitude. Glass micropipettes with approximately 50-μm cut tips were filled with normal Ringer's solution. They were used to record field potentials from the pial surface of the slice. After recording stable control field potentials for several minutes, the perfusate was changed to a Ringer's solution containing either kainic acid, folic acid, MTHF, or FTHF. Concentrations of these agents ranged from 10^{-8} M to 10^{-3} M; perfusion times ranged from 5 to 30 minutes. This was followed by a wash in normal Ringer's for a period of time equal to or greater than that of the drug treatment.

Kainic acid administered in concentrations greater than 10^{-8} M exhibited dose-dependent excitatory effects on the LOT-stimulated field potential. The lowest effective bath concentration, 5×10^{-7} M kainic acid, resulted in an increase in the amplitude of the population spike, indicating an increase in synchronous neuronal firing, with little effect on the slow wave representing the population excitatory postsynaptic potential (EPSP). At higher concentrations (Figure 1A), application of kainic acid resulted in an initial increase in amplitude of the population spike followed by a decrease in amplitude of the EPSP. As the EPSP approached the point of abolishment, the population spike increased to maximum size and then decreased and disappeared. These events appear to represent excitation followed by depolarization block. The action

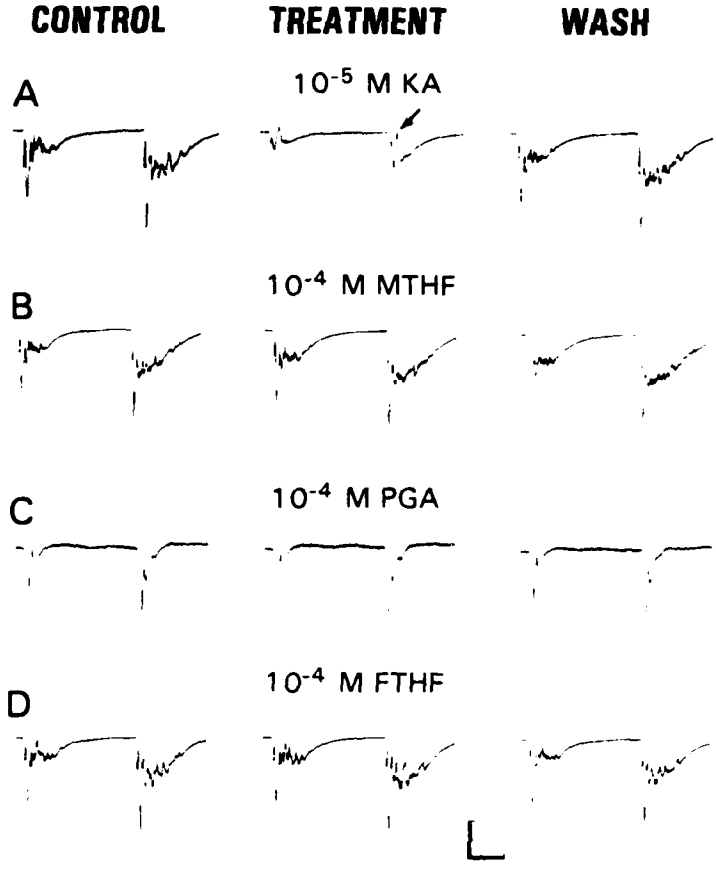

Figure 1. Effects of kainic acid and folates on field potentials stimulated by lateral olfactory tract (LOT) in rat olfactory cortex slices. Each trace shows two field potentials in response to double-shock stimulation of LOT. Initial downward deflection in each trace is beginning of excitatory postsynaptic potential. Rapid upward deflection is due to interruption of slow wave by population spike. Arrow in treatment condition A indicates population spike in second field potential. Spiking in late wave of field potential indicates asynchronous neural firing. Slices were superfused with (A) 10^{-5} M kainic acid (KA) for 5 minutes, (B) 10^{-4} M methyltetrahydrofolate (MTHF) for 15 minutes, (C) 10^{-4} M folic acid (PGA) for 20 minutes, and (D) 10^{-4} M formyltetrahydrofolate (FTHF) for 15 minutes. Duration of wash was equal to or greater than duration of treatment. MTHF and FTHF were prepared in oxygenated Ringer's just before use, and were protected from light during experiment. Vertical calibration bars: 1 mV for A, B, and D; 0.4 mV for C. Horizontal calibration bar: 20 mseconds for all traces.

of kainic acid was reversible if wash with normal Ringer's was initiated no later than the time of disappearance of the population spike. Kainic acid was toxic if left on the slice after the disappearance of the population spike.

In contrast to kainic acid, the MTHF (Figure 1B), folic acid (Figure 1C), and FTHF (Figure 1D) had little or no effect on the LOT-stimulated EPSP or population spike even when administered in concentrations as high as 1 mM. At 10^{-4} M or greater, these substances occasionally increased asynchronous spiking during the late stage of the EPSP. Since none of the folates reproduce the effects of kainic acid (even at 2000 times the concentration), it appears unlikely that the folates are physiological ligands for the kainic acid receptor.

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SCIENTIFIC SUPPORT DEPARTMENT

The Scientific Support Department is composed of three Divisions: Scientific Instrumentaton, Radiological Physics, and Radiation Sources.

During fiscal year 1981, the Scientific Instrumentation Division (SID) concentrated on six major areas of study:

Evaluation of various radiolabeled agents with specific affinity for muscarinic receptor

Production of fluorine-18 in the AFRRI TRIGA reactor for eventual incorporation into biologically active substances

Study of effects of cephalic compression on fetal cerebral blood flow and possible chronic injury

Study of membrane damage postirradiation, using electron paramagnetic resonance (EPR) techniques to assess and isolate the damage

Development of a biological dosimeter using the EPR analysis of calcified tissues

Although SID supervises AFRRI's electron microscopy unit and its gas chromatographic mass spectrometer (support spectrometers), SID's primary function continues to be radiobiological research. The Division uses radionuclides and sophisticated radiopharmaceuticals not only for *in vitro* studies of rodents but also for *in vivo* studies of rodents and canines. The *in vitro* work permits the rapid and more extensive study of radiation insult to biological models, thus setting the basis for later studies with more intricate, noninvasive imaging of a higher order animal model. These radiotracer studies help evaluate biological transport, metabolic events, and other physiological functions in experimental animal models. SID is also involved in using EPR to investigate the postirradiation damage to membranes.

The experimental models and radionuclide techniques developed or used in these areas of study provide the means of addressing radiobiological issues and medical problems of military significance. Specific radiolabeled muscarinic agents

are potentially useful for assessing the extent of damage from irradiation and/or chemical agents to a parasympathetic antonomic function. SID has undertaken an extensive collaborative research effort to develop a muscarinic radiopharmaceutical for this purpose.

One of the most rapidly developing noninvasive probes in nuclear medicine for assessing the quantity and quality of radiotracer data is positron emission computerized tomography (PECT). The advantage of PECT is the great number of specific radiolabeled biochemicals that can be used to assess biodistribution, metabolism, and excretion in radiobiological studies. SID has been investigating the production of fluorine-18, an important radionuclide for PECT studies, in AFRRI's TRIGA reactor.

One of SID's collaborative projects involves studying the effects of cephalic compression on fetal blood flow in specific vital organs. This project is medically relevant to the study of compression insult in the fetal model, and may also relate to compression insult to military personnel in combat.

Radiation-induced structural and biochemical aberrations leading to cellular dysfunction and death may be a manifestation of damage to the plasma membrane. SID is using EPR spectroscopy and sensitive nitroxide probes to investigate the membrane sites that are potentially more radiosensitive and thus more likely than others to contribute to cellular dysfunction or death.

Natural background ionizing radiation is known to produce stable, long-lived, free radicals in calcified tissues. EPR analysis of these free radicals has made possible the archeological dating of fossil bones. SID applies this technique to determining the radiation exposure doses for calcified tissues. The purpose of this study is to develop a biological dosimeter.

The Radiological Physics Division (RPD) provides dosimetry support for all radiation sources at AFRRI. Although it is primarily supportive, RPD provides a highly scientific function that requires extensive in-house research on dosimetry. Its main areas of study are (a) measurement of tissue-to-air ratios (TAR's); conversion of air doses to tissue doses using tissue-equivalent phantoms; (b) field mapping; measurement of dose distribution; and (c) the study of new dosimetry systems for adaptation to the AFRRI program.

Specific areas of dosimetry research by RPD included the establishment of a computer data base for cross sections and kerma factors necessary to evalute the biological and physical parameters used in neutron dosimetry. The data base consisted of 37 neutron energy groups and 21 gamma energy groups. In addition, numerous TAR determinations were made using the reactor spectra. These data, along with measurements of reactor spectra, allowed the revision and verification of the paired chamber equation coefficients in various protocols of irradiation. The paired chamber coefficients are used in reactor dosimetry to determine the dose in a mixed neutron-gamma field.

The Radiation Sources Division maintains and performs scheduled checks for quality control on AFRRI sources and accelerator (TRIGA reactor, cobalt-60 source, Theratron, Phillips Industrial X-ray unit, and LINAC). This ensures that AFRRI and outside investigators can compare their experiments quickly and accurately.

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EVALUATION OF THE RECEPTOR-BINDING RADIOPHARMACEUTICAL DIHYDROALPRENOLOL

Principal Investigators: W. A. Alter III, M. Grissom, J. Hill, and F. Vieras, *AFRRI*
W. Eckelman and J. Phillips, *George Washington University, Washington, DC*

This study was undertaken to investigate the use of receptor-binding radiopharmaceuticals as myocardial imaging agents. For imaging, it is desirable to have high ratios of heart/blood (H/B) and heart/lung (H/L) as well as homogeneous distribution throughout the myocardium. A β-adrenergic antagonist (H-3) dihydroalprenolol (DHA) was found to have a relatively high affinity (Ka = 1.2×10^8 M^{-1}) for rat ventricular muscle.

Chloralose-anesthetized dogs (n = 4) were chosen for the study of myocardial uptake and distribution of DHA, so that these results could be compared with those for simultaneously injected thallium-201 (Tl). Both the H/B and H/L ratios were higher for Tl (32.6 ± 10.0 and 7.9 ± 3.6, respectively) than for DHA (4.2 ± 1.1 and 0.34 ± 0.08, respectively).

Myocardium perfused by the left anterior descending coronary artery was rendered ischemic for 2 hours by ligation (n = 3) of this vessel, resulting in a marked reduction in uptake of both agents. Minimum values were measured in tissues obtained from the apical portion of the left ventricle where DHA was 35.2 ± 12.3% and Tl was 34.7 ± 13.6% of those values measured in normally perfused myocardium. Significant correlation ($r = 0.84$, $p < 0.001$) was seen between the values of these two agents within the entire ischemic region.

These results indicate that DHA is similar to Tl in its ability to discriminate between normal tissue and ischemic (or infarcted) tissue. Despite this good correlation, DHA does not have sufficiently high myocardial selectivity (relative to lung and blood) to be attractive as a parent structure for a gamma-labeled myocardial imaging agent.

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RADIOCHEMICAL SEPARATION OF FLUORINE-18 PRODUCED IN THE AFRRI TRIGA REACTOR

Principal Investigators: R. R. Eng and M. Grissom

One of the most promising areas of noninvasive determination of the distributive, metabolic, and excretory functions in biological systems is positron emission computerized tomography (PECT). Essentially, PECT measures the quality and quantity of radiotracer distribution in living systems. An important positron emitter used in PECT is fluorine-18.

Fluorine-18 has been produced in AFRRI's TRIGA reactor by means of an indirect nuclear chemical reaction: $^{6}Li(n,\alpha)T - {}^{16}O\ (T,n)^{18}F$. The target material, lithium-6 carbonate, is bombarded with fission neutrons, converting the lithium-6 to an energetic tritium atom (triton). The triton then bombards proximal ^{16}O atoms to produce fluorine-18. Since tritons are intermediate products, a significant amount of tritium activity is produced with the fluorine-18.

Before proceeding with any radiochemical synthesis with fluorine-18, the tritium must be removed. To accomplish this, a liquid chromatography procedure was developed. The irradiated lithium carbonate was dissolved with concentrated sulfuric acid, and the solution was then adjusted to pH 6.0 with aqueous ammonia. The 6.0-pH solution was passed through an alumina column and rinsed with three equal volumes of 6.0-pH aqueous solution (H_2SO_4-adjusted). The fluorine-18 was trapped on the alumina, and the tritium passed through the column. Concentrated NH_4OH solution was then passed through the column to remove the fluorine-18.

Not more than 3% tritium content was ever recorded. Thus, the problem of tritium contamination was solved by liquid chromatography techniques, so that radiochemical procedures could be developed.

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EFFECT OF CEPHALIC COMPRESSION ON CEREBRAL BLOOD FLOW OF THE FETUS

Principal Investigators: M. Grissom and R. R. Eng, *AFRRI*
W. O'Brian, S. Davis, and S. Golden, *Uniformed Services University of the Health Sciences*

Fetal cephalic compression always occurs during human labor. Despite the importance of this event, the effects of cephalic compression on the fetus are incompletely understood. Although the association of fetal bradycardia with cephalic compression is well recognized, the general influence of compression on fetal well-being is uncertain. Toward this end, we studied the effect of external cephalic compression on fetal pulse rate, aortic pressure, total cerebral blood flow, and regional distribution of cerebral blood flow in fetal lambs.

Ewes of mixed breed at 125 to 130 days of gestation were obtained. Cephalic compression was applied for 150 seconds at 200 torr. Physiological parameters were monitored for 5 minutes from the start of the compression. Then the ewes were sacrified and the fetuses removed.

With cephalic compression, an initial bradyardia was observed (168 bpm to 100 bpm), followed by a prolonged tachycardia (maximum at 255 bpm). Fetal mean aortic pressure was elevated throughout the study (55 to 75 torr). Although cardiac blood flow did not change, highly significant decreases were seen in cerebral blood flow (Figure 1). The mean blood flow of total brain fell from 154 to 27 ml/min/100 g of tissue. Mean cerebral vascular resistance increased from 0.40 to 31.2 torr/ml/min/100 g tissue.

In conclusion, marked alterations in fetal pulse rate, blood pressure, and cerebral blood flow associated with cephalic compression were observed in the near-term fetal lamb. The implications of these observations require further studies.

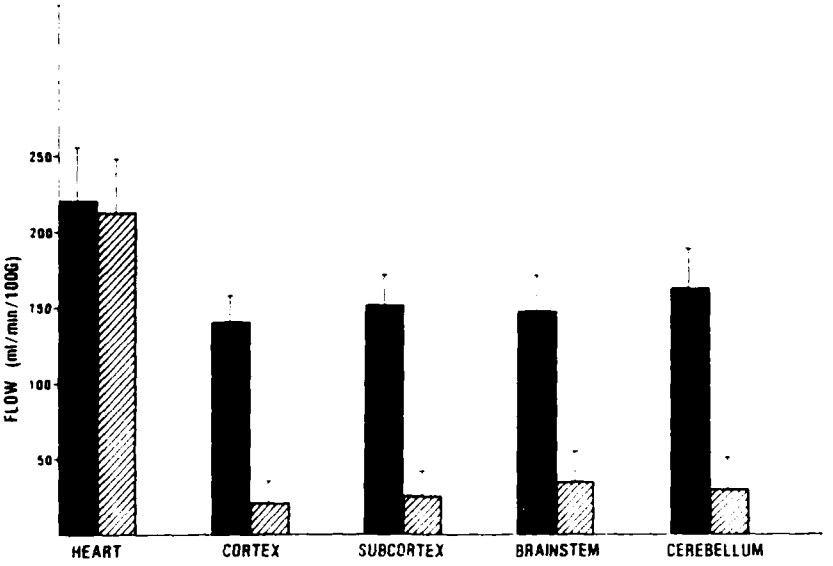

Figure 1. Organ blood flows ($\bar{X}$ ± SEM) in fetal lambs before (solid bars) cephalic compression and during (hatched bars) cephalic compression

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LOW-DOSE PARAMAGNETIC SIGNALS PRODUCED BY IONIZING RADIATION IN CALCIFIED TISSUES

Principal Investigators: M. J. McCreery, C. W. Swenberg, and J. J. Conklin

Ionizing radiation is known to produce several paramagnetic defects in calcified tissues. At least one of these defects is known to be stable for a minimum of 10^7 years, and it has been used for the archeological dating of fossil bones.

Using the technique of electron paramagnetic resonance (EPR), we have observed these radiation-induced defects in a variety of calcified tissues, including human tooth, rat tooth, bovine bone, and rat bone. Both stable and unstable signals were recorded, with the central line occurring at g = 2.0023.

Measurement of the intensity of these lines from tissues irradiated in air with a 130,000-Ci ^{60}Co gamma source or with electrons from a Varian linear accelerator yielded a linear dose-response relationship at doses from 10^2 to 10^6 rads. Signal-averaging techniques were necessary for accurate quantitation at exposures less than 500 rads, and they allowed the detection of doses as low as 13 rads.

The relationship of dose and signal intensity at low doses appeared to be hyperbolic, with saturation occurring at about 100 rads. The linear and nonlinear behaviors of the high-dose and low-dose domains, respectively, suggest that at least two types of stable paramagnetic centers are generated by ionizing radiation. The molecular nature of these defects is still unclear.

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A SPIN LABEL STUDY OF RADIATION-INDUCED DAMAGE IN MEMBRANES

Principal Investigators: M. J. McCreery, C. E. Swenberg, and W. A. Hunt

Radiation-induced structural and biochemical aberrations that lead to cellular dysfunction and death may be a manifestation of damage to the plasma membrane. Using nitroxide spin labels, the effects of ionizing radiation on both the lipid matrix and protein moieties have been investigated for rat erythrocytes and their isolated ghost membranes. The 5-, 12-, or 16-doxyl stearic acids and doxyl cholestane were incorporated into the membrane by dissolving the probe in chloroform, evaporating a coat of the spin label on the inside of a test tube, and then incubating it with the membranes for 20 minutes.

The stearic acid probes showed no detectable changes in their spectra for membranes exposed to $\leq$100,000 rads of ^{60}Co gamma. However, comparison of irradiated membranes with control membranes probed with cholestane showed small but significant changes in the spectra. Membrane proteins were labeled with 4-(2-bromoacetamido)-2,2,6,6-tetramethylpiperidinooxyl and 4-maleimido-2,2,6,6-tetramethylpiperidinooxyl by dissolving the probe in Pipes buffer at pH 6.8 and incubating it with membranes for 12 hours. Free probe was removed by repeated centrifugation.

The spectra of both probes revealed an immobilized and very mobile component. For the maleimide label, no difference in the spectra for irradiated samples and controls was detected. For the iodoacetamide probe, the EPR signal for the irradiated sample was reduced significantly more by $NiCl_2$ than for the nonirradiated membranes.

This enhanced reduction of signal intensity indicates that the labeled thiol groups are more exposed after irradiation. This result is consistent with the protrusions seen in electron microscopy of cells exposed to radiation. Order parameters of these spin labels and the results of saturation-transfer experiments using the maleimide probe also support these conclusions.

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